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(21) International Application Number: PCT/US98/08602 (22) International Filing Date: 28 April 1998 (28.04.98) (30) Priority Data: 60/044,256 28 April 1997 (28.04.97) US (71) Applicant (for all designated States except US): THE UNITED STATES OF AMERICA, represented by THE SECRETARY DEPARTMENT OF HEALTH & HUMAN SERVICES, THE NATIONAL INSTITUTES OF HEALTH [US/US]; Office of Technology Transfer, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852-3804 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): KELLEY, Michael, J. [US/US]; 8200 Gallery Court, Montgomery Village, MD 20886 (US). NAKAGAWA, Kazuhiko [JP/JP]; 1-12-9, Oonodai, Osakasayama, Osaka 589 (JP). DENT, Barry, Roy [NZ/NZ]; 1 Donald Crescent, Wellington 6005 (NZ). (74) Agent: SLATER, Stacey, C.; Klarquist, Sparkman, Campbell, Leigh & Whinston, LLP, One World Trade Center, Suite 1600, 121 S.W. Salmon Street, Portland, OR 97204-2988 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: CYCLIN DEPENDENT KINASE (CDK)4 INHIBITORS AND THEIR USE FOR TREATING CANCER (57) Abstract <p>Certain derivatives of acridones and benzothiadiazines have been found to have anti-cancer properties by virtue of their specific inhibition of the cyclin D dependant kinase CDK4. These molecules inhibit CDK4 activity more than they inhibit the activity of other such kinases (e.g. CDC2 and CDK2). This specificity results in an improved therapeutic index when used as drugs to treat susceptible cancers.</p>		

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CYCLIN DEPENDENT KINASE (CDK)4 INHIBITORS AND THEIR USE FOR TREATING CANCER

I. FIELD OF THE INVENTION

5 The present invention concerns compounds that inhibit cyclin-dependent kinases, particularly the cyclin-dependent kinase CDK4, and methods for treating cancers using such compounds.

II. BACKGROUND OF THE INVENTION

10 Physiology

 In a normal cell CDK4:cyclin D kinase holoenzyme phosphorylates the retinoblastoma protein (Rb) to form hyperphosphorylated retinoblastoma-phosphate (Rb-p). The hyperphosphorylation of retinoblastoma protein results in the release of Rb-p associated transcription factors that allow cell cycle
15 progression beyond the G1 checkpoint, thereby promoting cell proliferation (Schrr et al., U.S. Patent No. 5,723,313, (1998)).

 The p16 gene (also known as CDKN2, MST1, and CDK4I) encodes the protein p16^{INK4A}, which inhibits the cyclin-dependent kinase (CDK)4:cyclin D complex (Serrano, et al., Nature 366: 704-7 (1993)). Defects in the
20 p16/CDK4:cyclinD/Rb pathway may lead to tumor formation. Genetic alteration or over expression of CDK4 and CyclinD1 has been observed in various tumor cell types. In addition, alterations of p16 have been described in various histologic types of human cancers including retinoblastoma, astrocytoma, melanoma, leukemia, breast cancer, head and neck squamous cell carcinoma,
25 malignant mesothelioma, and lung cancer (Kamb et al., Science 264: 436-40 (1994); Noborie et al., Nature 368: 753-56 (1994); Walker et al., Cancer Res. 55: 20-3 (1995) and Nakagawa et al., Oncogene 11: 1843-51 (1995)).

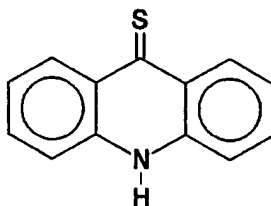
Acridones and Benzothiadiazines

30 Acridones and benzothiadiazines (BTDs) are classes of known cyclic aryl compounds. Certain known acridones or BTDs have pharmacological effects. For example, BTDs have been investigated as diuretics (See de Tullio et al., *J. Med. Chem.* Fajans and Floyd (*Ann. Rev. Med.* 30:313-329, 1982)

disclose the use of "diuretic benzothiadiazine, e.g. trichlormethiazide" as a hyperglycemic in the treatment of insulinomas. Fajans and Floyd, however, do not teach the use of BTDs to affect cancers directly. The prior art, as understood, does not appear to teach the use of BTDs for their direct
5 antineoplastic effect in the specific inhibition of CDK4 dependent tumors.

Particular acridones and acridines are known. For example, (C₁₈H₁₉N₃O₂-HCl) has been mentioned in a paper concerned with the anti-tumor activity of linear tri-cyclic carboxamides (Palmer et al., J. Med. Chem (US) 31 (4) pgs.707-721, 1988). Interestingly, the Palmer et al. paper states that this
10 compound is "inactive" (page 711, column 1, paragraph 3).

The basic *thio*acridone ring structure was described in DeLeenheer et al., *J. Pharm. Sci.* 60:1238-1239, 1971, and is shown below.



20 1-nitro-9-acridone, 1-nitro-10-(3-N,N-dimethylaminopropyl)-9-acridone, 1-amino-2,4-diethylthio-9-acridone and a number of acridine derivatives have been disclosed by Weltrowski et al. (*Pol. J. Chem Technol.* 56:77-82, 1982). This paper, however, deals exclusively with the synthesis of
25 nitroacridines and does not discuss any biological activity or mechanism of biological action. But, the title of the Weltrowski article refers to tumor inhibition, and the footnote states that the work was supported by the Polish National Cancer Program.

30 III. SUMMARY OF THE INVENTION

The present invention concerns acridones, benzothiadiazines and derivatives thereof that are useful for treating cancers. The invention also concerns methods for using these compounds as CDK4 inhibitors to treat cancers.

There are a number of dreadful and relatively common cancers that have been shown to involve alterations in p16. These cancers include lung cancer, breast cancer, melanoma, leukemia, retinoblastoma, astrocytoma, head and neck squamous cell carcinoma and malignant mesothelioma. Expression of normal p16 protein in tumor cells with alterations of p16 results in restoration of cell-cycle regulation, decreased cell growth and decreased tumorigenicity *in vivo*. Because the only known function of p16 is inhibition of CDK4 kinase activity, cancers with alterations of p16, including those listed above, are likely to be sensitive to CDK4 inhibitors. Prior inhibitors of cyclin-dependent kinases, such as flavopiridole, staurosporin, and UCN-01, inhibit CDC2 and CDK2 as well as the intended target, CDK4. This lack of specificity produces pathological side effects, such as bone marrow and gastrointestinal toxicities, and limits their clinical application.

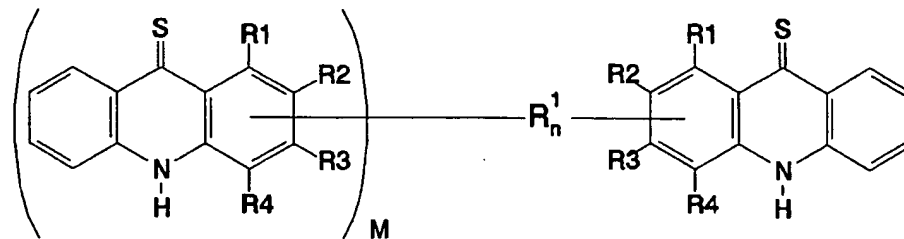
As a result, there is a need for drugs for treating CDK4 sensitive neoplasms that minimize toxic side effects caused by concomitant inhibition of CDC2 and CDK2. The compounds claimed in this application inhibit CDK4 to a far greater extent than CDC2 or CDK2 and therefore satisfy this need.

One example of a novel compound of the present invention is 3-amino-9-thio(10H)-acridone. This compound and others can be used to form therapeutic compositions. One embodiment of such a composition comprises a therapeutically effective amount of a compound selected from the group consisting of a benzothiadiazine, a thioacridone, or mixtures thereof. The compound has an IC_{50} for CDK4 of less than about 10 μM , preferably from about 1 μM to about 7 μM , an IC_{50} for CDC2 of greater than about 60 μM , preferably greater than about 100 μM , an IC_{50} for CDK2/A of greater than about 100 μM , an IC_{50} for CDK2/E of greater than about 80 μM , and preferably greater than about 100 μM .

The specificity of the compounds for inhibiting CDK4 can be expressed as a ratio of the IC_{50} values for other enzymes relative to CDK4. Such compositions typically comprise a compound selected from the group consisting of a benzothiadiazine, a thioacridone, or mixtures thereof, the compound having an IC_{50} ratio for CDC2:CDK4 of greater than about 8.5, typically greater than about 20, preferably greater than about 60; an IC_{50} ratio for CDK2/A:CDK4 of

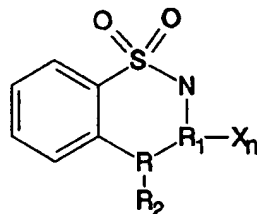
greater than about 14, typically greater than about 20, and preferably greater than about 60; and an IC_{50} ratio for CDC2/E:CDK4 of greater than about 11.5, typically greater than about 20, and preferably greater than about 60.

The invention also provides a composition comprising an effective
5 amount of a compound according to Formula 1



where m is 0 or 1, $n=m$, R_1 - R_4 are independently selected from the group consisting of H, $-NH_2$ and lower alkoxy, where with $m=1$ one of R_1 - R_4 is an amine bonded to R' to form an arylamide,

10 or Formula 2



15

where R and R_1 are independently carbon or nitrogen, where if R_1 =carbon X is hydrogen, halogen, aryl or alkoxy, and R_2 is selected from the group consisting
20 of lower alkyl and aryl amino. The composition also can comprise mixtures of compounds satisfying Formula 1 and/or Formula 2. The composition can further include, without limitation, additives selected from the group consisting of carriers, diluents, excipients, diagnostics, direct compression buffers, buffers, stabilizers, fillers, disintegrates, flavors, colors, and mixtures thereof.

25 A method for inhibiting the growth of living cells also is described. The method comprises providing a compound selected from the group consisting

of a benzothiadiazine, a thioacridone, or mixtures thereof, as described above. An effective amount of the compound, a mixture of compounds, or a composition comprising the compound or mixture of compounds, is administered to a subject to inhibit the growth of living cells.

5

IV. BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1(A)-1(I) are dose-response curves showing the effect of Compound 5 on various cancer cell lines in culture.

FIG. 2 shows mean plots of data from FIGS. 1A-1I, wherein the left-hand mean plot is of GI_{50} data, the middle mean plot is of TGI data, and the right-hand mean plot is of LC_{50} data.

FIGS. 3(A)-3(I) are dose-response curves showing the effect of Compound 7 on various cancer cell lines in culture.

FIG. 4 shows mean plots of data from FIGS. 3A-3I, wherein the left-hand mean plot is of GI_{50} data, the middle mean plot is of TGI data, and the right-hand mean plot is of LC_{50} data.

FIGS. 5(A)-5(I) are dose-response curves showing the effect of Compound 8 on various cancer cell lines in culture.

FIG. 6 shows mean plots of data from FIGS. 5A-5I, wherein the left-hand mean plot is of GI_{50} data, the middle mean plot is of TGI data, and the right-hand mean plot is of LC_{50} data.

FIGS. 7(A)-7(I) are dose-response curves showing the effect of Compound 4 on various cancer cell lines in culture.

FIG. 8 shows mean plots of data from FIGS. 7A-7I, wherein the left-hand mean plot is of GI_{50} data, the middle mean plot is of TGI data, and the right-hand mean plot is of LC_{50} data.

FIGS. 9(A)-9(I) are dose-response curves showing the effect of Compound 6 on various cancer cell lines in culture.

FIG. 10 shows mean plots of data from FIGS. 9A-9I, wherein the left-hand mean plot is of GI_{50} data, the middle mean plot is of TGI data, and the right-hand mean plot is of LC_{50} data.

FIGS. 11(A)-11(I) are dose-response curves showing the effect of

Compound 3 on various cancer cell lines in culture.

FIG. 12 shows mean plots of data from FIGS. 11A-11I, wherein the left-hand mean plot is of GI_{50} data, the middle mean plot is of TGI data, and the right-hand mean plot is of LC_{50} data.

5

V. DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

DEFINITIONS

Particular terms and phrases used herein typically have the meanings set forth below. These definitions are provided solely for convenience and should not be interpreted to limit the invention to a scope less than that known to a person of ordinary skill in the art.

10

"3-ATA" means 3-amino-9-thio(10H)-acridone.

15

"BTD" means benzothiadiazine.

"Neoplasm" and "cancer" both refer to any cell or tissue wherein growth and cell division have become uncoupled from the normal regulatory constraints of the cell cycle to produce a pathological state.

20

"Tumor" is any neoplasm and includes both solid and non-solid neoplasms.

25

"Inhibitory concentration" or " IC_{50} " means the drug concentration at 50% inhibition of kinase activity (μM).

"Therapeutically effective anti-neoplastic amount" means an amount sufficient to prevent advancement, or to cause regression of, a neoplasm.

30

"CDK4" and "CDK4/A" refer to the CDK4:cyclin D1 kinase holoenzyme.

"CDK4 inhibitor" refers to compounds that inhibit the kinase activity of CDK4.

"CDK4 inhibition" refers to inhibition of the kinase activity of CDK4.

5 "CDK2", when used alone, refers to both CDK2:Cyclin A and to CDK2:Cyclin E

"CDC2" and "CDC2/A" refer to CDC2:Cyclin A holoenzyme.

10 "CDK2/A" refers to CDK:Cyclin A holoenzyme.

"CDK2/E" refers to CDK2:Cyclin E holoenzyme.

 "Cancers specifically inhibited by CDK4 inhibitors" means all
15 neoplastically transformed cells and tissues, the growth and/or cell cycle of which is affected by a CDK4 inhibitor.

 A cell "susceptible to CDK4 inhibitors" or "susceptible to CDK4 inhibition" is a cell for which CDK4 inhibitors alter growth or cell cycle.

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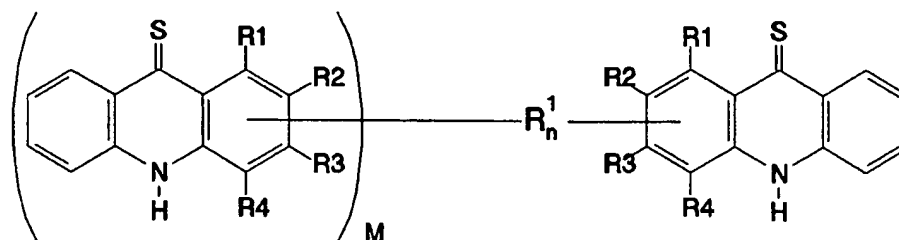
 "Specific inhibition" or "specific inhibitory activity" of the compounds of the invention means that the compounds inhibit CDK4 to a greater extent than they inhibit CDC2 or CDK2.

25 "Lower alkyl" means a single-bonded branched or unbranched hydrocarbon chain having from about one to about ten carbon atoms, including all position and stereoisomers.

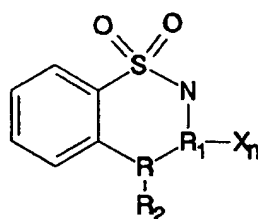
COMPOUNDS

30 Compounds of the present invention satisfy either Formula 1 (acridone-like structures) or Formula 2 (benzothiadiazine-like structures) below.

FORMULA 1



FORMULA 2



5
10 With reference to Formula 1, m is 0 or 1, and $n = m$. R_1 - R_4 are independently selected from the group consisting of H, $-NH_2$ and lower alkoxy. With $m=1$, at least one of R_1 - R_4 is an amine and R' is bonded to the amine to form an arylamide.

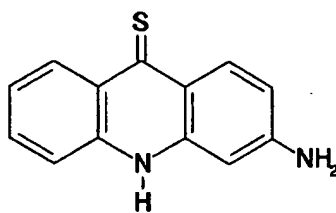
15 With reference to Formula 2, R and R_1 are independently carbon or nitrogen. If R_1 =carbon X is hydrogen or halogen. R_2 is selected from the group consisting of lower alkyl and aryl amino.

20 Compounds according to both Formula 1 and 2 show specific inhibitory activity against CDK4. This inhibition may be due to inhibition of formation of the CDK4:cyclinD kinase holoenzyme or to competitive binding of the inhibitor with the kinase substrate or to ATP-dependent competitive effects or some other interaction.

Structural formulas for particular compounds of the invention are provided below as Compounds 1-6.

COMPOUND 1

5

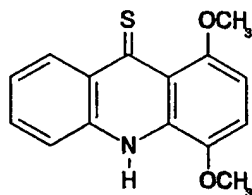


3-Amino-10H-acridine-9-thione

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COMPOUND 2

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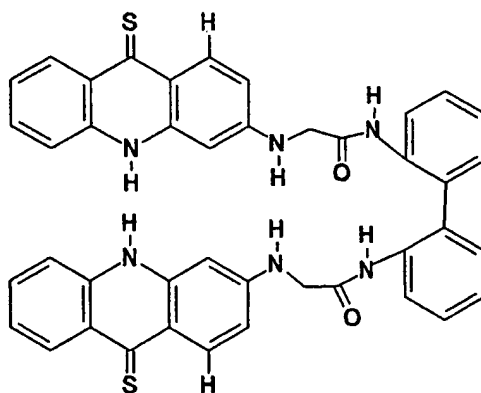


1,4-Dimethoxy-10H-acridine-9-thione

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COMPOUND 3

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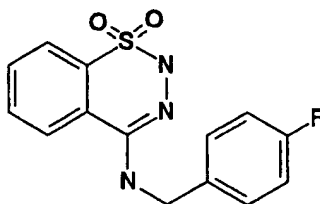


30 2,2'-Biphenyldiamine, bis[N,N'-[3-(amidomethylamino)-10H-acridine-9-thione]]

- 10 -

COMPOUND 4

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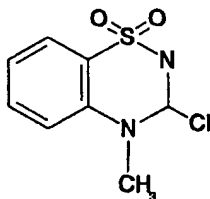


4-(4-Fluorobenzylamino)-1,2,3-benzothiadiazine-1,1-dioxide

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COMPOUND 5

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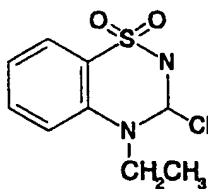


3-Chloro-4-methyl-4H-benzo[e][1,2,4]thiadiazine 1,1-dioxide

20

COMPOUND 6

25



3-Chloro-4-ethyl-4H-benzo[e][1,2,4]thiadiazine 1,1-dioxide

SYNTHESIS OF COMPOUNDS

The compounds of the invention were obtained from and are maintained at the Drug Synthesis and Chemistry Branch, National Cancer Institute. Syntheses of related compounds are known in the literature. For example, the following references described the syntheses of certain related

compounds: Pascal de Tullio et al., "3- and 4- Substituted 4*H*-Pyrido[4,3-*e*]-1,2,4-thiadiazine 1,1-Dioxides as Potassium Channel Openers: Synthesis, Pharmacological Evaluation, and Structure--Activity Relationships," *J. Med. Chem.*, Vol. 39, pp. 937-948 (1996); Bernard A. Dumaitre et al., U.S. Patent No. 5,604,237; Hamprecht et al., U.S. Patent No. 4,075,004; Magatti U.S. Patent No. 4,468,396; Brian D. Palmer et al., "Potential Antitumor Agents. 54. Chromophore Requirements for in Vivo Antitumor Activity Among the General Class of Linear Tricyclic Carboxamides," *J. Med. Chem.*, Vol. 31, pp. 707-712 (1988); N. Dodic et al., "Synthesis and Activity Against Multidrug Resistance in Chinese Hamster Ovary Cells of New Acridone-4-Carboxamides," *J. Med. Chem.*, Vol. 38, pp. 2418-2426 (1995); Marek Weltrowski et al., "Research on Tumour Inhibiting Compounds, Part LXX, Reactions of 1-Nitroacridines with Ethanethiol," *Polish Journal of Chemistry*, pp. 77-82 (1982).

15 COMPOSITIONS

Compounds satisfying either Formula 1 or 2 above may be formulated as pharmacological compositions containing a therapeutically effective anti-neoplastic amount of the compound(s). Such compositions may further comprise, without limitation, inert carriers, diluents, excipients, diagnostics, direct
20 compression buffers, buffers, stabilizers, fillers, disintegrates, flavors, colors, other materials conventionally used in the formulation of pharmacological compositions and mixtures thereof.

METHOD

25 The method of the present invention comprises administering to a subject a therapeutically effective anti-neoplastic amount of a compound, mixture of compounds, or composition or compositions comprising the compound or compounds, to effect a change in the physiology of a neoplasm. One of ordinary skill in the art will realize that the therapeutically effective anti-neoplastic amount
30 may vary. Anti-tumor agents generally are dosed as mass-per-unit-body surface area of the subject. It currently is believed that a therapeutically effective anti-neoplastic amount of the disclosed compounds may be from about 1 µg to about

10 g per m² of body surface area, more preferably from about 1 mg to about 900 mg per m² of body surface area. Moreover, it typically is desirable to provide as large a dose as a subject will tolerate.

The compound(s) or compositions may be administered by any number of methods including, but not limited to, intravenously, topically, orally, intramuscularly, subcutaneously, intraperitoneally. Currently, intravenous and oral administration are considered the preferable routes of administration.

BIOLOGICAL METHODS AND RESULTS

Tables 1 and 2 provide IC₅₀ data for compounds representative of the present invention. These tables demonstrate that the IC₅₀ value of compounds according to the present invention for CDK4 generally is less than about 10 μM, and preferably is less than about 7 μM. The best compound, solely in terms of its IC₅₀ value for CDK4, is compound 5 with an IC₅₀ of 1.1 μM. But, compounds 7 and 8 also have IC₅₀ values of less than 2 μM, namely 1.4 μM and 1.7 μM respectively.

The compounds of the present invention also are quite specific for inhibition of CDK4. This is reflected in the IC₅₀ ratios reported in Tables 1 and 2, with the IC₅₀ for CDK4 being the denominator in the ratio e.g., (IC₅₀ CDC2)/(IC₅₀ CDK4). Thus, the lower the IC₅₀ is for CDK4 and the higher it is for the other complexes, the more specific the compound is for CDK4.

The CDC2/A:CDK4 ratios in Tables 1 and 2 range from about 8 to greater than 72. The best compound with respect to specificity between CDK4 and CDC2 is compound 7, with an IC₅₀ for CDK4 of 1.4 μM, an IC₅₀ for CDC2 of >100 μM, and an (IC₅₀ CDC2):(IC₅₀ CDK4) of >71.5.

Compound 3 (3-ATA) has an IC₅₀ for CDK4 of 6.8 μM, an IC₅₀ for CDC2 of 60 μM, and an (IC₅₀ CDC2):(IC₅₀ CDK4) of 8.8.

Compound 4 has an IC₅₀ for CDK4 of 2.2 μM, an IC₅₀ for CDC2 of >100 μM, and an (IC₅₀ CDC2):(IC₅₀ CDK4) of >45.

Compound 5 has an IC₅₀ for CDK4 of 1.1 μM, an IC₅₀ for CDC2 of >70 μM, and an (IC₅₀ CDC2):(IC₅₀ CDK4) of >63.6.

Compound 6 has an IC₅₀ for CDK4 of 5.0 μM, an IC₅₀ for CDC2 of

> 100 μ M, and an (IC₅₀ CDC2):(IC₅₀ CDK4) of >71.5.

Compound 8 has an IC₅₀ for CDK4 of 1.7 μ M, an IC₅₀ for CDC2 of > 100 μ M, and an (IC₅₀ CDC2):(IC₅₀ CDK4) of >58.8.

IC₅₀ and IC₅₀ ratio data for other kinases are summarized in Tables 1 and 2 below.

Compounds satisfying Formulas 1 and 2 have been subjected to biological assays to determine inhibition of the cyclin dependent kinases CDK4, CDC2, CDK2/A and CDK2/E. The experimental procedures for these biological methods and assays are provided below in the Examples. Results of these assays for representative compounds are provided below in Tables 1 and 2.

TABLE 1

Formula Name	IC ₅₀ value (μ M)						
	CDK4/D1	CDC2/A	Ratio CDC2A: CDK4	CDK2/A	Ratio CDK2/A: CDK4	CDK2/E	Ratio CDK2/E: CDK4
Compounds structurally related to 3-ATA							
Formula 3	6.8	60	8.8	>100	>14.7	80	11.8
Formula 4	2.2	>100	>45	>100	>45	>100	>45
Formula 5	1.1	70	63.6	>100	>91	>100	>91

TABLE 2

Formula Name	IC ₅₀ value (μM)							
	CDK4/D1	CDC2/A	Ratio CDC2A: CDK4	CDK2/A	Ratio CDK2/A: CDK4	CDK2/E	Ratio CDK2/E: CDK4	
5	Compounds structurally related to BTB (NSC645787)							
	Formula 6	5.0	>100	>20	>100	>20	>100	>20
10	Formula 6	1.4	>100	>71.5	>100	>71.4	>100	>71.4
15	Formula 7	1.7	>100	>58.8	>100	>58.8	>100	>58.8

20 An IC₅₀ of 10 μM is generally considered effective for these compounds, but effectiveness should be considered in the light of specificity for CDK4.

EXAMPLES

25 The following examples are provided to illustrate certain features of the invention and are not meant to limit the invention to any particular embodiment.

Example 1

30 This example describes in detail how the compounds of the invention were identified and tested to determine their specific inhibitory activity against cyclin dependent kinases. Essentially, the methods of this example include three stages: (1) determining which cell lines contain p16 alterations, (2) determining which drugs are most active against p16 altered cells, and (3) determining the CDK4 kinase inhibitory activity of selected, screened compounds.

METHODS

Cell lines, compounds, and *in vitro* sensitivity testing.

Exponentially growing cultures of the nine non-small cell lung, eight melanoma, eight renal, eight breast, seven colon, six brain, six leukemia, six ovarian, and two prostate cancer cell lines from the NCI drug screen panel were used. Compounds were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute. *In vitro* antitumor activity of compounds was determined using a sulforhodamine-B assay in the 60 human cancer cell lines of the NCI drug screen panel.

Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and DNA sequence analysis of p16.

Approximately 1.5×10^5 tumor cells were washed with PBS, lysed in 100 μ l proteinase K solution [200 mg/ml, 50 mM Tris-HCl (pH8.5), 1 mM EDTA(pH8.0), and 0.5% Tween, 20], and incubated at 50°C for 4 h. One microliter of this lysate was used as template in a 10 μ l PCR for each of seven oligonucleotide primer pairs which span the coding region and splice junctions of exons 1 and 2 of p16 twice. *Sma*I-digested (for primer pair 2D) or undigested PCR products were subjected to SSCP. The presence of bands with an abnormal migration pattern was confirmed by repeating PCR-SSCP at least once prior to extraction of the band, cloning into pT7Blue(R) T-vector (Novagen, Madison, WI), and DNA sequence analysis by the dideoxy chain termination method using SequenaseTM (US Biochemical, Cleveland, OH). The presence of intact genomic DNA was confirmed by amplification of a 536-bp fragment of the β -globin gene. The p16 sequence published by Okamoto et al. (GenBank accession number L27211) was used as reference for DNA and amino acid numbering.

Reverse Transcription (RT)-PCR and Southern blot hybridization analyses of p16. Total RNA was isolated from 1×10^6 cells of each cell line using an RNA isolation kit (5' prime 3' prime, Inc., Boulder, CO), RT-PCR was performed for the p16 gene as previously described. PCR products were separated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with a 388-bp p16 exon 1 genomic fragment defined by oligonucleotides 2F and 1108R. Expression of the glyceraldehyde-3-phosphate

(GAPDH) gene was examined to assure the presence of intact mRNA in each sample by addition of a gene-specific oligonucleotide, G3PD-2R (5'-GATACATGACAAGGTGCGGC-3') to the reverse transcriptase reaction followed by 40 cycles of PCR (30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C using oligonucleotides, G3PD-1F (5'TCGTGGAAGGACTCATGACC-3') and G3PD-1R (5'ACATGGCAACTGTGAGGAGG-3').

Immunoblot analysis. Cells (1×10^7) were washed with PBS, resuspended in 0.4ml of lysis buffer [50 mM Tris-HCl (pH7.4), 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P40, 50 mM NaF, and 1 mM PMSF], and centrifuged at 14,000 rpm for 20 min at 4°C. The protein concentration of the supernatant was determined using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Fifty micrograms of total protein were mixed with an equal volume of 2X sample buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% (w/v) SDS, 0.005% bromophenol blue, and 5% 2-mercaptoethanol], loaded on a 14% Tris-glycine gel, and subjected to electrophoresis at 125 V for 90 min in 1X running buffer (25 mM Tris-base, 192 mM glycine, and 0.1% SDS). The separated proteins were transferred to a nitrocellulose membrane at 25 V for 2 h in transfer buffer (12 mM Tris-base, and 96 mM glycine, 20% methanol). After 30 min incubation at room temperature in blocking solution (1X PBS, 5% powdered dry milk, and 1% BSA), the membrane was incubated at 4°C with 1:1000 dilution of polyclonal anti-human p16 antiserum (PharMingen, San Diego, CA) overnight, rinsed 5 times with PBS, incubated with a mixture of 40 μ l 125 I-Protein A (> 30 mCi/mg) in 20 ml blocking solution at 4°C for one hour, washed again with PBS, air dried for 15 min, and subjected to autoradiography.

COMPARE analysis. The COMPARE algorithm was performed. For the identification of agents with differential activity, "G150" values of 0 and 1 were used for p16-normal and for p16-altered cell lines, respectively. p16-altered cell lines were those with biallelic deletion, intragenic mutation, or transcriptional suppression of p16 and p16-normal cell lines were those without these abnormalities. Pearson correlation coefficients were calculated by the SAS procedure PROC CORR (SAS Institute Inc., Cary, NC).

GST fusion proteins. Full length p16 cDNA from cell lines

containing intragenic mutations (NCI-H69, MDA-MB-435, UACC-257, and DU-145) were produced by RT-PCR using oligonucleotides MK52

(5'CGTGAATTCAAGCTTCCTCTCTGGTTCTTTCAATCGGG-3') and MK68 (5'GATGGGATCCCGGCGGGGAGCAGC-3'), cloned into pGEX-5X-1

5 plasmid (Pharmacia Biotech, Piscataway, NJ) and sequenced. A GST-Rb fusion plasmid encoding the larger "pocket" domain of Rb was used and GST-fusion proteins were expressed in *E. coli* (DH5 α) and purified using glutathione sepharose (Pharmacia Biotech, Piscataway, NJ) according to manufacturers recommendations.

10 ***In vitro* kinase assay.** Seventy-two hours after infection of 1×10^7 Sf9 cells with baculovirus containing a human CDK gene and/or a cyclin gene, cells were lysed in 250 μ l of lysis buffer [50 mM HEPES (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 5 ig/ml of aprotinin, 5 μ g/ml of leupeptin, 0.1 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mM sodium
15 orthovanadate], centrifuged, and lysates stored at -70°C. Five microliters of CDK:cyclin lysate were mixed with test compounds in 40 μ l of kinase buffer (200 mM Tris-HCl, pH 8.0, 100 mM MgCl₂, 10 mM EGTA) and incubated at 30°C for 30 min. About 400 ng of purified GST-Rb fusion protein and 5 μ Ci of γ -[³²P]ATP were added to the mixture and incubated at 30°C for 15 min.
20 Reactions were stopped by the addition of 250 μ l of IP buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl, 0.5% NP-40) and 15 μ l glutathione sepharose. After one hour incubation at 4°C, sepharose beads were washed four times with IP buffer, mixed with 18 μ l of 2X sample buffer and electrophoresed on an 8% Tris-glycine gel (Novex, San Diego, CA) at 125 V for 90 min. Equal recovery
25 of GST-Rb fusion protein was confirmed by Coomassie blue staining prior to autoradiography.

CDK4 binding assay. Sf9 cells (1×10^7) were co-infected with baculovirus containing a cloned human CDK4 gene and/or a cyclin D1 gene in 12.5 ml of Grace's insect medium (Paragon, Baltimore, MD) containing 10%
30 FBS. After 40 h, cells were washed and placed in 5 ml of methionine-free medium containing 200 μ Ci/ml of [³⁵S]methionine (1000 Ci/mmol) for 4 h, followed by lysis in 250 μ l. Cleared cell lysate (10 μ l) was incubated with 400

ng of wildtype or mutant GST-p16 fusion proteins using the same conditions as the *in vitro* kinase assay. After a 30 min incubation, GST-p16 fusion protein was separated using glutathione sepharose according to manufacturer's recommendations, and electrophoresed on a 14% Tris-glycine gel (Novex, San Diego, CA). The gel was stained using Coomassie blue, dried, and autoradiography was performed. Equal recovery of GST-p16 fusion protein was confirmed by Coomassie blue staining. To test the effect of compounds on p16 binding to CDK4, 100 μ M of each compound was incubated with CDK4:cyclin D1 lysate for 30 min prior to adding GST-p16 fusion protein.

10

RESULTS

Characterization of the p16 status of the cell lines of the NCI drug screen panel. To detect genetic alternations of p16 in the 60 cell lines of the NCI drug screen panel, polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis was performed for exons 1 and 2 of the p16 gene using genomic DNA. Exon 3, which encodes only four amino acids, was not examined as mutations limited to exon 3 have not been described. Among the 60 cell lines, 29 cell lines were found to lack amplifiable genomic sequences of one or both exons, indicative of a biallelic deletion involving p16. The presence of amplifiable genomic DNA in each sample was confirmed by amplification of a 536 bp fragment of the β -globin gene. Eight of the 60 cell lines contained a reproducible abnormally migrating SSCP band. DNA sequence analysis of clones of these eight abnormally migrating SSCP fragments revealed alteration of the primary sequence in each. One of these eight cell lines, HL-60, had two sites of sequence variation in exon 2 of p16, one of which was a common polymorphism at codon 148 (A148T). This polymorphism, which does not affect p16 function, was also present in the colon carcinoma cell line, KM12. Additional sequence variants not known to be polymorphisms were observed in seven (12%) of the 60 cell lines. HL-60 contained a nonsense mutation at codon 80 and HCT-116 contained a one bp insertion at codon 22-23, which results in a frameshift at codon 22 and termination after codon 42. Both of these mutations were reasoned to cause loss of p16 function. Three cell lines (MDA-

MB-435, MDA-N, and M14) contained the same splice site mutation [T to C substitution at nucleotide 2 of intron 1 (I1+2^{T-C})], and 2 cell lines (UACC-257 and DU-145) had distinct missense mutations. The splice site mutation resulted in aberrant splicing creating a shortened mRNA that had deletion of codons 28 to 50. The functional effect of the splice site and missense mutations was assessed by measuring the binding of GST-p16 fusion proteins to CDK4. Binding of mutant GST-p16 fusion proteins (I1+2^{T-C}, D84Y, and P81L) to CDK4 was 3.2%, 4.9%, and 34% of the binding ability of normal p16, respectively ($p < 0.0001$ for each comparison, 2-tailed Student t-test). Thus, 36 of 60 (60%) cell lines of the NCI drug screen panel contained a genetic alteration (homozygous deletion or intragenic mutation) of p16 that disrupted the function of p16^{INK4A}.

To detect non-genetic alterations associated with loss of p16 function, p16 mRNA and protein expression were examined. Using RT-PCR and subsequent Southern blot hybridization analyses, p16 mRNA expression was undetectable in 41 of 60 (68%) cell lines examined, including 11 of 24 (46%) without detectable genetic alteration. The amplified p16 cDNAs in two cell lines (MDA-MB-435 and MDA-N) were smaller than expected, consistent with altered mRNA splicing as a result of the I1+2^{T-C} mutation. p16 mRNA was not detected in the third cell line (M14) with this splice site mutation. A protein of 16 kd was detected in 17 of the 60 (28%) cell lines by Western blot analysis using p16 polyclonal antiserum. The cell line with a nonsense mutation (HL60) expressed p16 mRNA but not p16 protein. The two cell lines with missense mutations (UACC-257 and DU-145) expressed both mRNA and protein. In UACC-257, a protein smaller than 16 kd was detected, perhaps the result of altered susceptibility to proteolysis of p16^{P81L}. A protein of 16 kd was detected in two cell lines with the splice site mutation (MDA-MB-435 and MDA-N) but was absent in the third cell line with the I1+2^{T-C} mutation, M14. In each cell line, absent or altered p16 protein could be attributed to mutation or transcriptional suppression. In total, 47 of the 60 (78%) cell lines of the NCI drug screen panel had an alteration of p16.

Comparison of p16 status with growth inhibitory activity. To identify compounds more active against p16-altered cells than p16-normal cells, the p16 status of the 60 cell lines was matched to the growth inhibitory (GI_{50}) activity of the compounds of the NCI drug screen program and ranked according to Pearson correlation coefficients using the COMPARE algorithm. The growth inhibitory activity of cephalostatin 1, a disteroidal alkaloid extracted from the marine worm, *Cephalodiscus gilchristi*, correlated best with p16 status ($r=0.599$). The growth inhibitory activity of five related compounds [cephalostatins 7, 9, 8, 4 and 3 were also positively correlated with p16 status ($r=0.504$, 0.493 , 0.491 , 0.461 , and 0.458 , respectively). Bryostatin 1, a protein kinase C activator isolated from the marine bryozoan, *Bugula neritina*, had a correlation coefficient of 0.469 .

Aliquots of 26 of the 40 compounds with the highest Pearson correlation rankings were available for further *in vitro* analysis. These compounds were assessed for CDK4:cylin D kinase inhibitory activity using baculovirus-expressed human CDK4 and cyclin D1, and a GST-Rb fusion protein as substrate. Six of the 26 compounds examined inhibited phosphorylation of Rb protein by CDK4:cyclin D1 complex with IC_{50} values ranging from 6.8 to more than $100 \mu M$. No inhibition of GST-Rb phosphorylation by CDK4:cyclin D1 was observed in the presence of the other 20 compounds at concentrations up to $100 \mu M$. The most potent inhibitor was 3-amino-9-thio(10H)-acridone (3-ATA; Formula 3) with an IC_{50} of $6.8 \mu M$, a value similar to the mean GI_{50} ($30 \mu M$) observed for this compound in the 2 day growth assay of the NCI drug screen. Cephalostatin 1, which has potent antitumor activity *in vitro* (ED_{50} 10^{-7} to $10^{-9} \mu g/ml$), had an IC_{50} for CDK4:cyclin D1 of $20 \mu M$ and bryostatin 1 had no inhibitory activity at the highest concentration examined ($100 \mu M$).

Characterization of 3-ATA. To examine the specificity of 3-ATA inhibitory activity for CDK4:cyclin D1 kinase, we performed *in vitro* kinase assays using baculovirus-expressed human CDC2:cyclin A, CDK2:cyclin A, and CDK2:cyclin E complexes. 3-ATA was a less potent inhibitor of CDC2 and CDK2 kinase activities with IC_{50} values at least nine-fold higher compared to the IC_{50} for CDK4. The addition of $100 \mu M$ 3-ATA decreased the binding of CDK4

to normal p16 by 70% in the p16-CDK4 binding assay ($p < 0.0001$, 2-tailed Student t-test), suggesting that 3-ATA may be acting by a mechanism similar to p16. In the CDK4 kinase assay, the addition of exogenous ATP (0 to 600 μM) did not alter the inhibitory activity of 3-ATA, suggesting that 3-ATA was not
5 competing with ATP. Thus, 3-ATA appears to inhibit cyclin-dependent kinase activity by a mechanism distinct from that of the flavone L86827 and butyrolactone I, which are known to compete with ATP.

Identification of CDK4-specific inhibitors. To identify compounds in the NCI drug screen that may have a similar mechanism of action as 3-ATA,
10 the pattern of growth inhibitory activity (GI_{50}) of 3-ATA with the GI_{50} of all previously tested compounds as compared. Six compounds not previously examined for CDK4 kinase inhibitory activity had similar patterns of growth inhibitory activity with correlation coefficients greater than 0.6. Among these six, two benzothiadiazine (BTD) compounds (Compound 6) and NSC 645788)
15 inhibited CDK4:cyclin D1 kinase activity *in vitro* with IC_{50} 's (5.0 and 17 μM , respectively) similar to the IC_{50} of 3-ATA (6.8 μM).

An additional 45 compounds with structural similarity to 3-ATA and (Compound 6) were available for analysis. Nineteen of these compounds inhibited CDK4 kinase activity with IC_{50} 's ranging from 1.1 to more than 100
20 μM . Four compounds, 2 structurally related to 3-ATA (Compound 4) and NSC 645153), and 2, Compound 7 and Compound 8, were more potent CDK4 kinase inhibitors than the parent compounds. Compound 4, Compound 7, and Compound 8 also had no CDC2 or CDK2 kinase inhibitory activity at concentrations up to 100 μM . However, two of these compounds, Compound 4
25 and Compound 7, did not inhibit p16^{INK4A} binding to CDK4, suggesting that their mechanism of inhibition of CDK4 kinase activity is distinct from 3-ATA.

Example 2

This example describes a method for treating cancer using the
30 compounds of the invention. Thioacridones or benzothiadiazines satisfying Formulas 1 and 2 above are obtained that specifically inhibit CDK4:cyclin kinase such that these compounds have an IC_{50} for CDK4 that is smaller than their IC_{50}

for CDC2 or CDK2. These compounds are administered intravenously or orally to humans at a dose of between 1 μ g and 10 grams, preferable between 1mg and 900 mg per m² of body surface of the patient. The compounds also can be mixed with at least one additive selected from the group consisting of carriers, diluents, excipients, diagnostics, direct compression buffers, buffers, stabilizers, fillers, disintegrates, flavors, colors, and mixtures thereof to form pharmaceutical compositions. The compositions are administered intravenously or orally to humans at a dose of between 1 μ g and 10 grams, preferable between 1mg and 900 mg per m² of body surface of the patient.

CELL LINE DATA

Compounds of the present invention have been subjected to the drug screening procedure employed by the National Cancer Institute for the screening of drugs having possible anticancer utility. The screening procedure uses a diverse, disease-oriented panel consisting of different human tumor cell lines organized into disease-specific subpanels. The compounds of the present invention were tested over a range of concentrations for cytotoxic or growth-inhibitory effects against cell lines comprising the panel. The subpanels represented diverse histologies (leukemias, melanomas, and tumors of the lung, colon, kidney, breast, ovary, and brain). The tests produced individual dose-responses, one for each cell line (i.e., one for each example), and the data are disclosed in dose-response curves, e.g., FIGS. 1(A)-1(I). The data provided by these dose response curves are summarized using a mean-graph format, e.g., FIG. 2.

To produce data for the mean-graph format, a compound concentration that produced a target level response was calculated for each cell line. Three different response parameters were evaluated. The first response parameter was the growth inhibition ("GI₅₀"). GI₅₀ is the concentration of compounds made according to the present invention that produced an apparent 50% decrease in the number of tumor cells relative to the appropriate control (not exposed to the compounds of the present invention) at the end of the incubation period.

The second response parameter was the total growth inhibition

("TGI"). TGI is the concentration at which the number of tumor cells remaining at the end of the incubation period substantially equal the number of tumor cells existing at the start of the incubation period.

The third response parameter was the lethal concentration ("LC₅₀").

- 5 LC₅₀ is the concentration of compounds made according to the present invention that caused an apparent 50 percent reduction in the number of tumor cells relative to the appropriate control (not exposed to the compounds of the present invention) at the start of the incubation period.

10 In a typical GI₅₀ mean graph the relative position of the vertical reference line along the horizontal concentration axis was obtained by averaging the negative log₁₀GI₅₀ values for all the cell lines tested against the compound. Horizontal bars were then plotted for the individual negative log₁₀GI₅₀ values of each cell line relative to the vertical reference line. The GI₅₀ graph thus provides a characteristic fingerprint for the compound, displaying the individual cell lines
15 that are proportionately more sensitive than average (bars extending to the right of the reference line) or proportionately less sensitive than average (bars extending to the left of the reference line). The length of a bar is proportional to the difference between the log₁₀GI₅₀ value obtained with the particular cell line and the mean (represented by the vertical reference line).

- 20 The data obtained using the cell line procedures referred to above are provided by FIGS. 1-12. This data shows that the compounds of the present invention inhibit the growth of living cells.

WE CLAIM:

1. 3-amino-9- thio(10H)-acridone.
2. An antineoplastic composition comprising a therapeutically
5 effective amount of a compound selected from the group consisting of a
benzothiadiazine, a thioacridone, or mixtures thereof, the compound having an
IC₅₀ for CDK4 of less than about 10 μM.
3. The antineoplastic composition of claim 2 wherein the compound
10 has an IC₅₀ for CDC2 of more than about 60 μM.
4. The antineoplastic composition of claim 3 wherein the compound
has an IC₅₀ for CDK2/A of more than about 100 μM.
5. The antineoplastic composition of claim 4 wherein the compound
15 has an IC₅₀ for CDK2/E of more than about 80 μM.
6. The antineoplastic composition of claim 2 wherein the compound
has an IC₅₀ for CDK4 of less than about 2.5 μM.
20
7. The antineoplastic composition of claim 3 wherein the compound
further has an IC₅₀ for CDC2 of greater than about 100 μM.
8. The antineoplastic composition of claim 7 wherein the compound
25 further has an IC₅₀ for CDK2/A of greater than about 100 μM.
9. The antineoplastic composition of claim 8 wherein the compound
further has an IC₅₀ for CDK2/E of greater than about 100 μM.
10. An antineoplastic composition comprising a compound selected
30 from the group consisting of a benzothiadiazine, a thioacridone, or mixtures
thereof, the compound having an IC₅₀ ratio for CDC2:CDK4 of greater than

about 8.5.

11. The antineoplastic composition of claim 10, wherein the compound has an IC_{50} ratio for CDK2/A:CDK4 of greater than about 14.

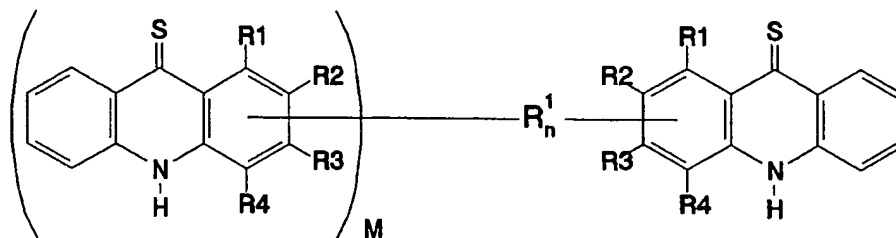
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12. The antineoplastic composition of claim 11, wherein the compound has an IC_{50} ratio for CDC2/E:CDK4 of greater than about 11.5.

13. The antineoplastic composition of claim 10 wherein the compound
10 has an IC_{50} ratio for CDC2:CDK4 of greater than about 20, an IC_{50} ratio for CDK2/A:CDK4 of greater than about 20, and an IC_{50} ratio for CDC2/E:CDK4 of greater than about 20.

14. The antineoplastic composition of claim 10 wherein the compound
15 has an IC_{50} ratio for CDC2:CDK4 of greater than about 60, an IC_{50} ratio for CDK2/A:CDK4 of greater than about 60, and an IC_{50} ratio for CDC2/E:CDK4 of greater than about 60.

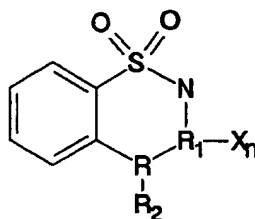
15. An antineoplastic composition comprising an effective amount of a
20 compound according to Formula 1



where m is 0 or 1, $n = m$, R_1 - R_4 are independently selected from the group consisting of H, $-NH_2$ and lower alkoxy, where with $m=1$ one of R_1 - R_4 is an amine bonded to $R=$ to form an arylamide,

Formula 2

5



where R and R₁ are independently carbon or nitrogen, where if R₁=carbon X is
10 hydrogen or halogen, and R₂ is selected from the group consisting of lower alkyl
and aryl amino,

and mixtures of compounds satisfying Formula 1 and/or Formula 2, wherein the
compounds have an IC₅₀ for CDK4 of less than about 10 μM.

15 16. The composition according to claim 15 wherein the compound has
an IC₅₀ for CDC2 of more than about 60 μM.

17. The composition according to claim 15 wherein the compound has
an IC₅₀ for CDK2/A of greater than about 100 μM.

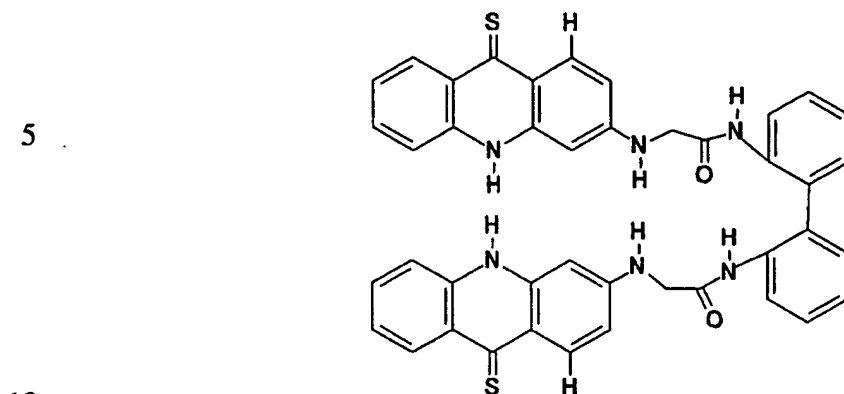
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18. The composition according to claim 15 wherein the compound has
an IC₅₀ for CDK2/E of greater than about 80 μM.

19. The composition according to claim 15 and further comprising
25 additives selected from the group consisting of carriers, diluents, excipients,
diagnostics, direct compression buffers, buffers, stabilizers, fillers, disintegrates,
flavors, colors, and mixtures thereof.

20. The composition according to claim 15 wherein the effective
30 amount of the compound is sufficient to provide from about 1 mg to about 900
mg per m² body surface area of a subject treated with the composition.

21. The composition according to claim 13 comprising



22. The composition according to claim 15 where, with respect to Formula 1, $m=n=0$.

15

23. The composition according to claim 22 where at least one of R_1 - R_4 is an amine, the remainder of R_1 - R_4 being hydrogen.

24. The composition according to claim 22 comprising 3-amino-9-thio(10H)-acridone.

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25. The composition according to claim 22 where at least one of R_1 - R_4 is lower alkoxy.

26. The composition according to claim 22 where at least two of R_1 - R_4 are lower alkoxy.

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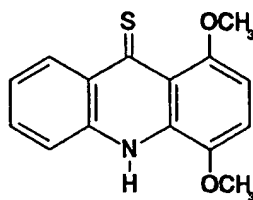
27. The composition according to claim 22 where at least two of R_1 - R_4 are methoxy.

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28. The composition according to claim 22 comprising

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29. The composition according to claim 15 where, with respect to
10 Formula 2, R is nitrogen.

30. The composition according to claim 27 where R₂ is a lower alkyl.

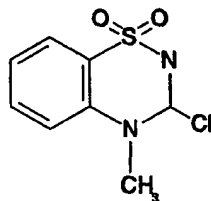
31. The composition according to claim 28 where R₂ is selected from
15 the group consisting of methyl and ethyl.

32. The composition according to claim 27 where X is halogen.

33. The composition according to claim 28 where X is halogen.
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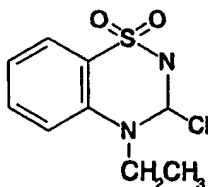
34. The composition according to claim 31 comprising

25



35. The composition according to claim 31 comprising

5



36. The composition according to claim 15 where the compound has
10 an IC_{50} for CDK4/D1 of less than about 10 μM .

37. The composition according to claim 15 where the compound has
an IC_{50} for CDK4/D1 of from about 1 μM to about 7 μM .

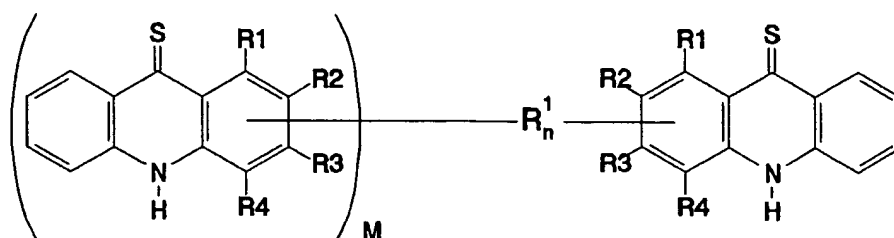
15 38. The composition according to claim 35 where the compound has
 IC_{50} values for CDC2/A and CDK2/A of greater than about 60 μM .

39. A method for inhibiting the growth of living cells, comprising:
providing a compound selected from the group consisting of a
20 benzothiadiazine, a thioacridone, or mixtures thereof, the compound having an
 IC_{50} for CDK4 of less than about 10 μM , an IC_{50} for CDC2 of more than about
60 μM , an IC_{50} for CDK2/A of more than about 100 μM , and an IC_{50} for
CDK2/E of more than about 80 μM ; and
administering an effective amount of the compound to inhibit the
25 growth of living cells.

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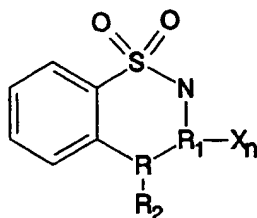
40. A method for inhibiting the growth of living cells, comprising:
 providing a compound selected from the group consisting of a
 benzothiadiazine, a thioacridone, or mixtures thereof, the compound having the
 compound having an IC_{50} ratio for CDC2:CDK4 of greater than about 8.5, an
 5 IC_{50} ratio for CDK2/A:CDK4 of greater than about 14, and an IC_{50} ratio for
 CDC2/E:CDK4 of greater than about 11.5; and
 administering an effective amount of the compound to inhibit the
 growth of living cells.

- 10 41. The method according to claim 40 where the compound satisfies
 Formula 1



- where m is 0 or 1, $n = m$, R_1 - R_4 are independently selected from the group
 consisting of H, $-NH_2$ and lower alkoxy, where with $m=1$ $R=$ is an arylamide
 15 bonded to one of R_1 - R_4 which is an amine,
 or Formula 2

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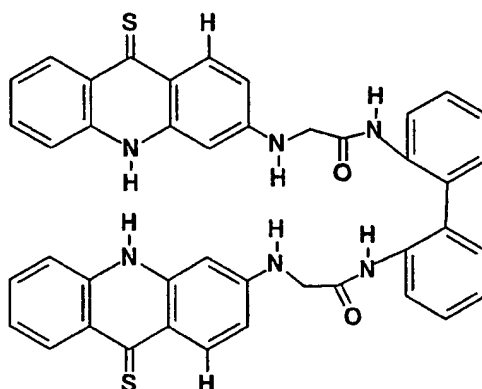


- where R and R_1 are independently carbon or nitrogen, where if R_1 =carbon X is
 25 hydrogen or halogen, and R_2 is selected from the group consisting of lower alkyl

and aryl amino, and mixtures of compounds satisfying Formula 1 and/or Formula 2.

42. The method according to claim 39 where the step of providing a compound comprises providing a composition comprising the compound, and further comprising additives selected from the group consisting of carriers, diluents, excipients, diagnostics, direct compression buffers, buffers, stabilizers, fillers, disintegrates, flavors, colors, and mixtures thereof.

43. The method according to claim 41 comprising



44. The method according to claim 41 where, with respect to Formula 1, $m=n=0$.

45. The method according to claim 44 where at least one of R_1 - R_4 is an amine, the remainder of R_1 - R_4 being hydrogen.

46. The method according to claim 44 comprising 3-amino-9-thio(10H)-acridone.

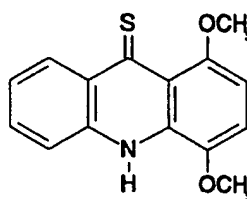
47. The method according to claim 41 where at least one of R_1 - R_4 is lower alkoxy.

48. The method according to claim 41 where at least two of R_1 - R_4 are lower alkoxy.

49. The method according to claim 41 where at least two of R_1 - R_4 are methoxy.

50. The method according to claim 49 comprising

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51. The method according to claim 41 where, with respect to Formula 2, R is nitrogen.

52. The method according to claim 51 where R_2 is lower alkyl.

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53. The method according to claim 52 where R_2 is selected from the group consisting of methyl and ethyl.

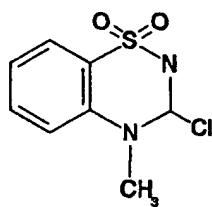
54. The method according to claim 51 where X is halogen.

25

55. The method according to claim 52 where X is halogen.

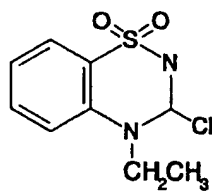
56. The method according to claim 51 comprising

5



57. The method according to claim 51 comprising

10



1/47

Fig. 1 (A)

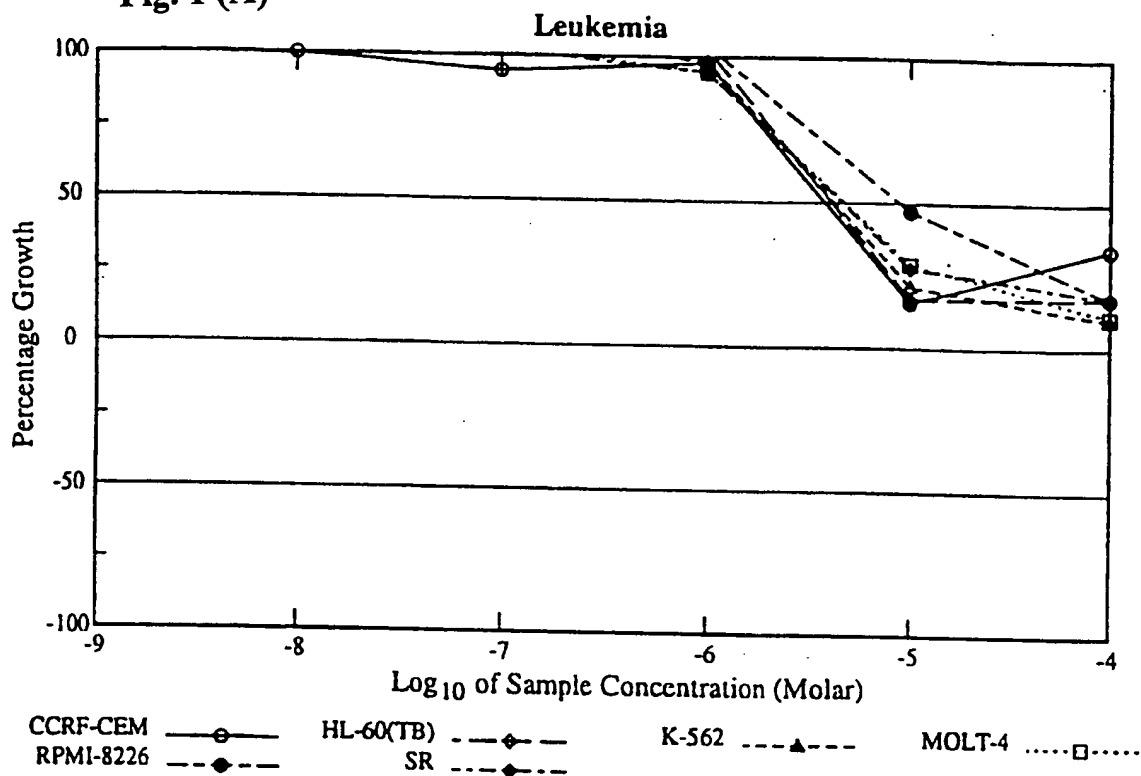
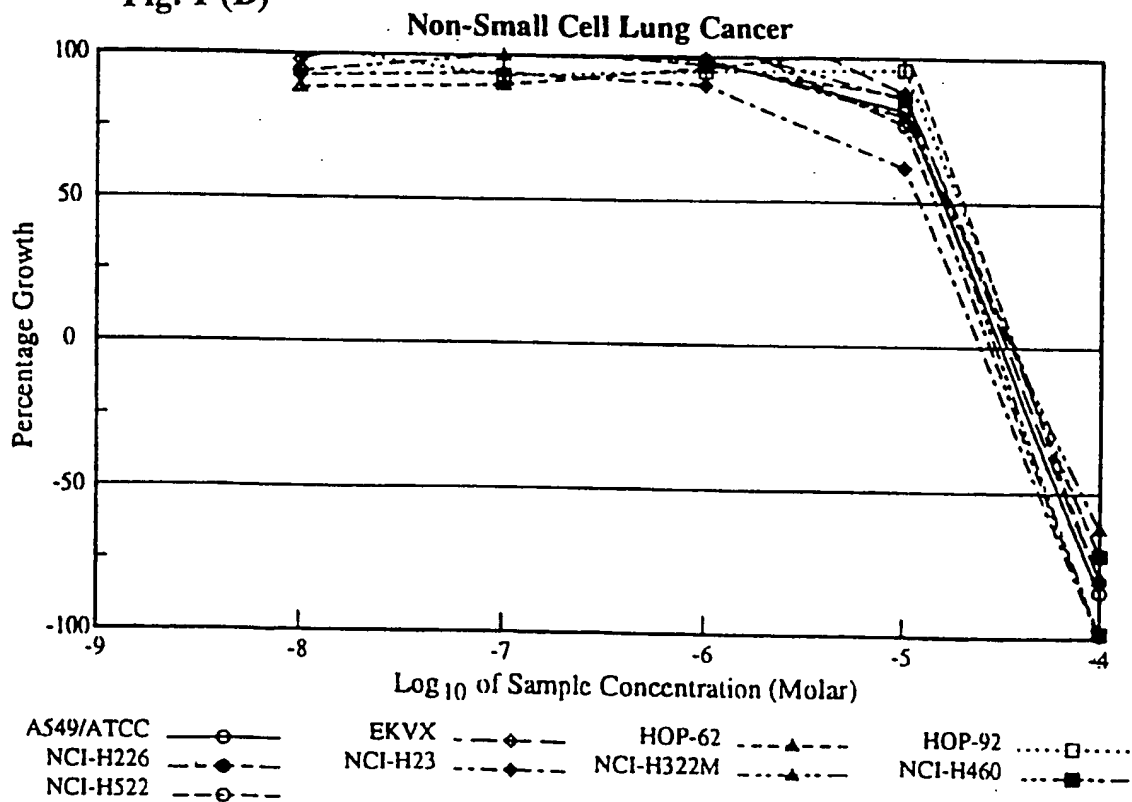


Fig. 1 (B)



2/47

Fig. 1 (C)

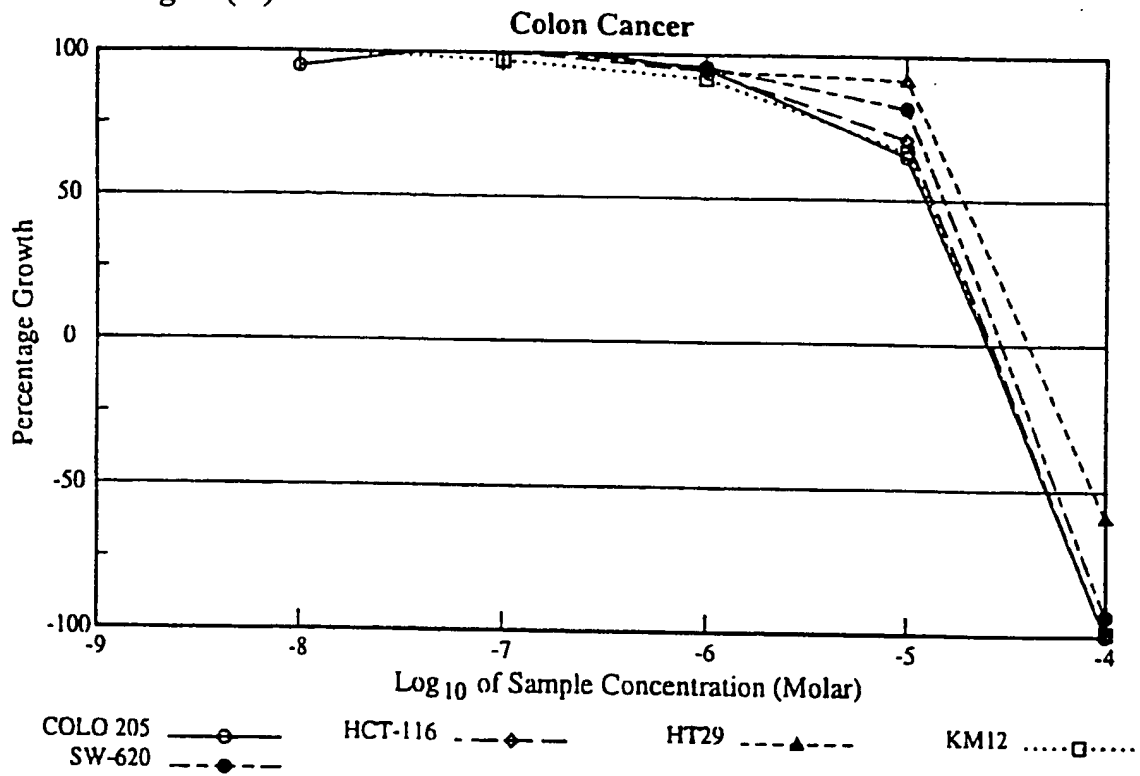
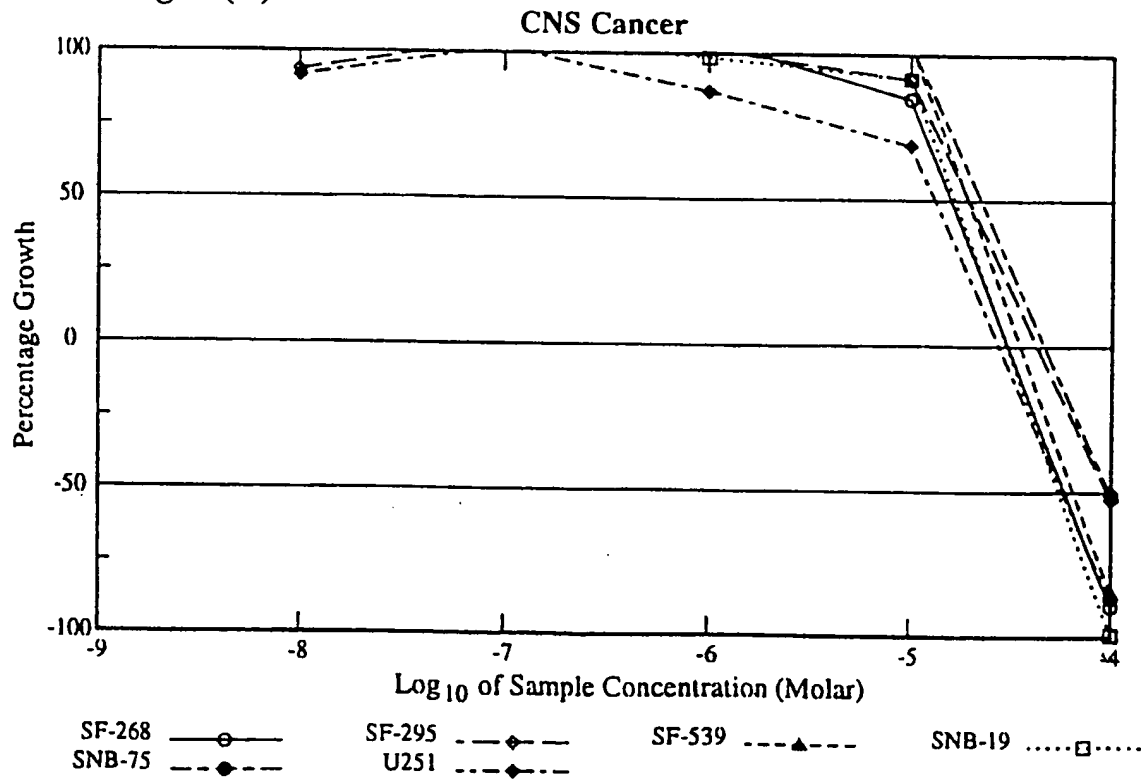


Fig. 1 (D)



3/47

Fig. 1 (E)

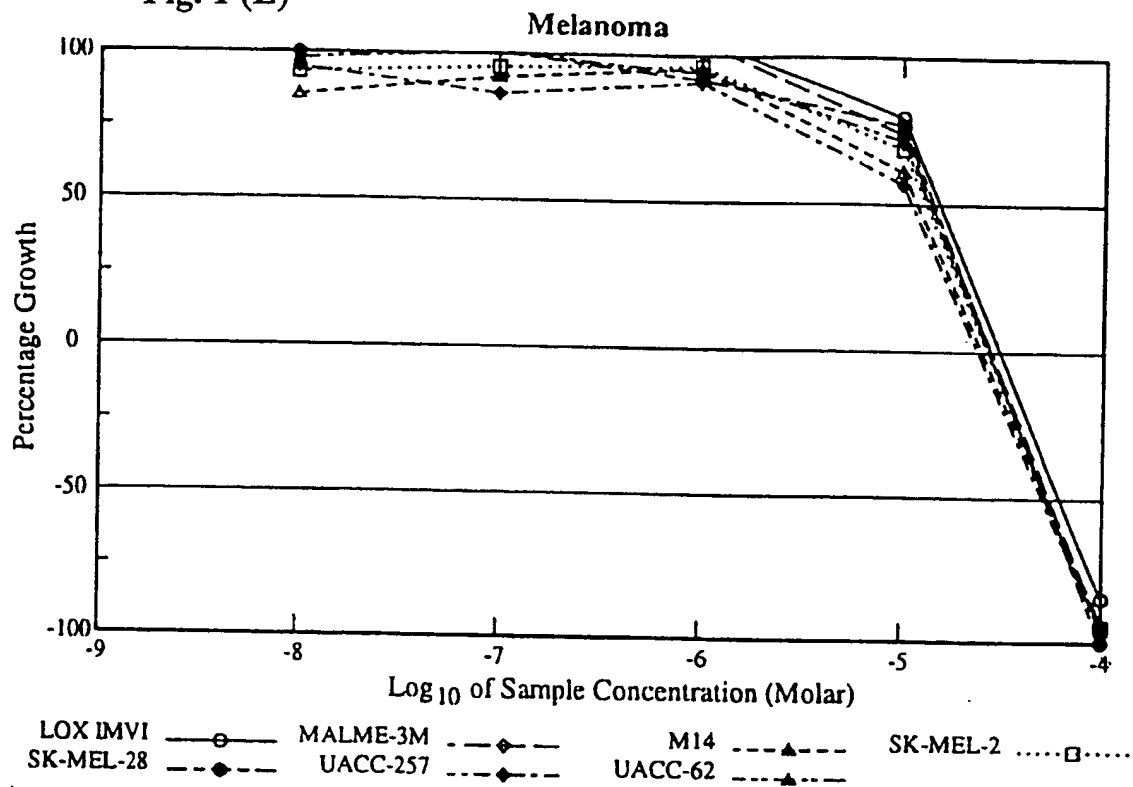
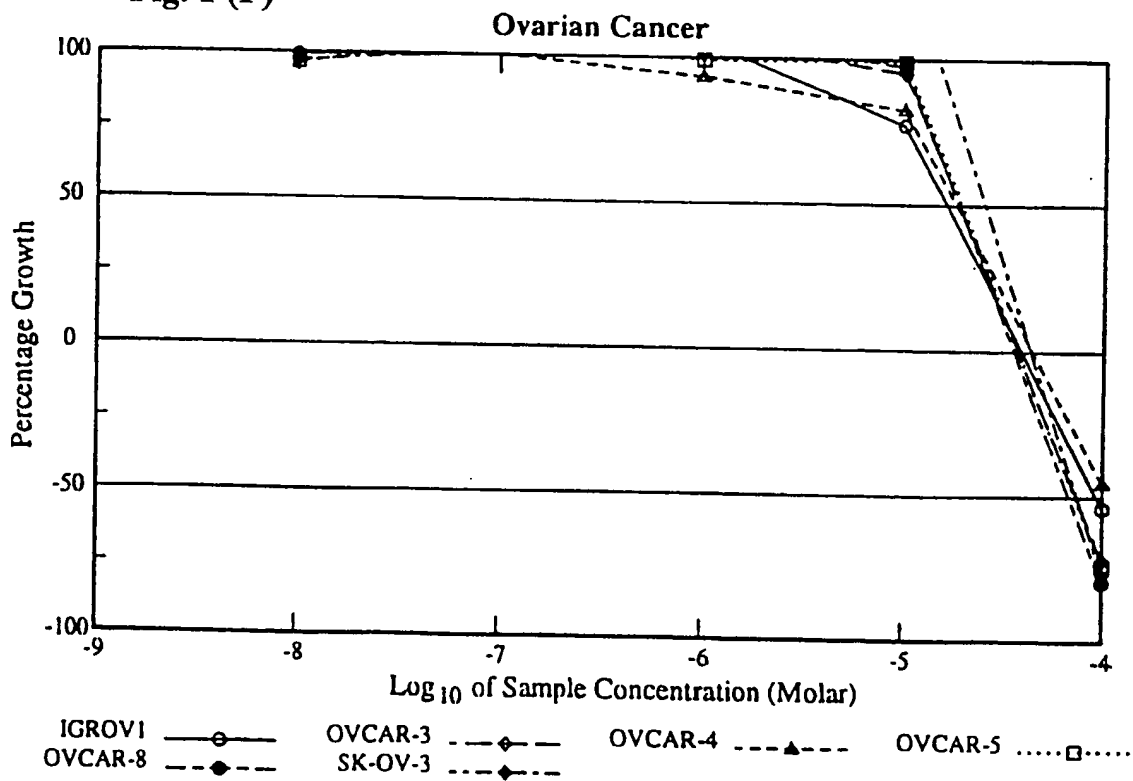


Fig. 1 (F)



4/47

Fig. 1 (G)

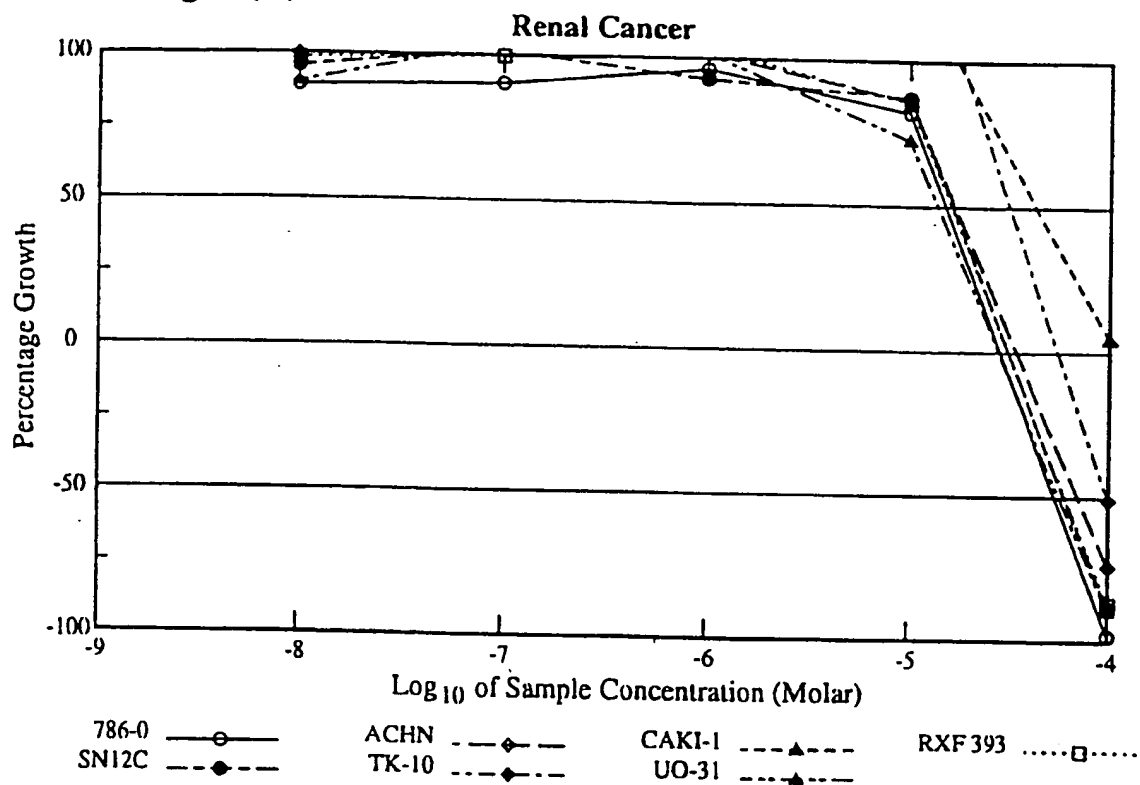
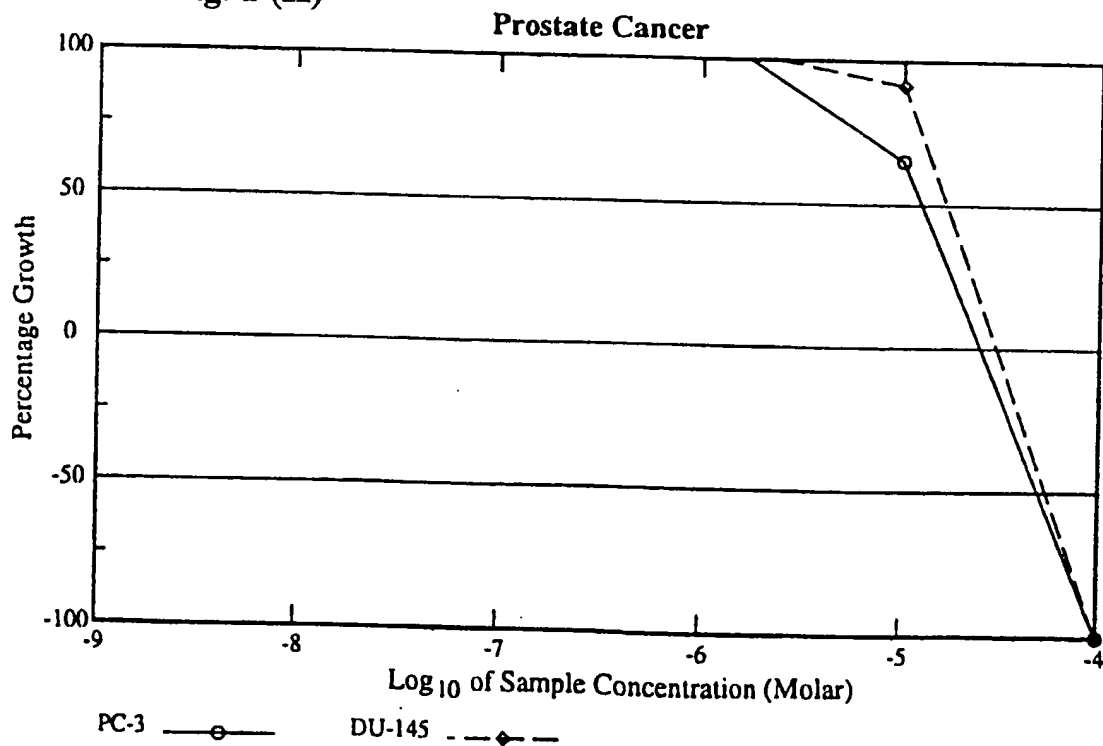


Fig. 1 (H)



5/47

Fig. 1 (I)

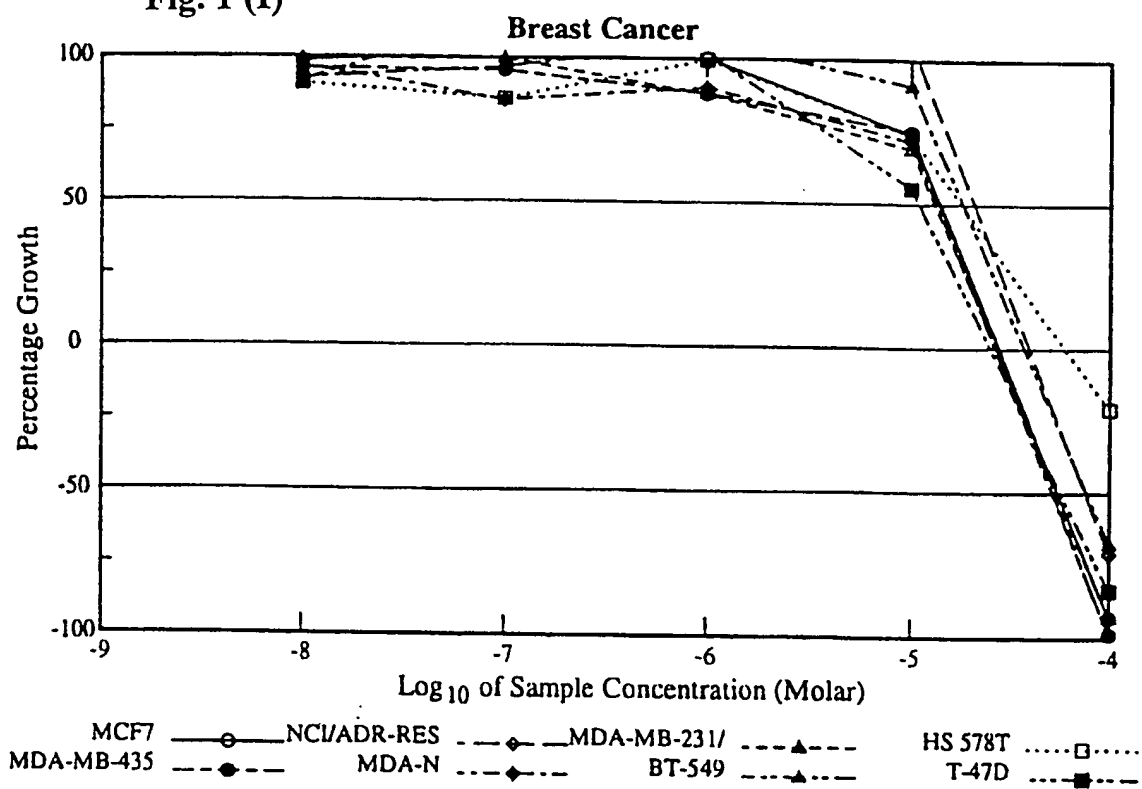


Fig. 2 (Sheet 1 of 3)

6/47

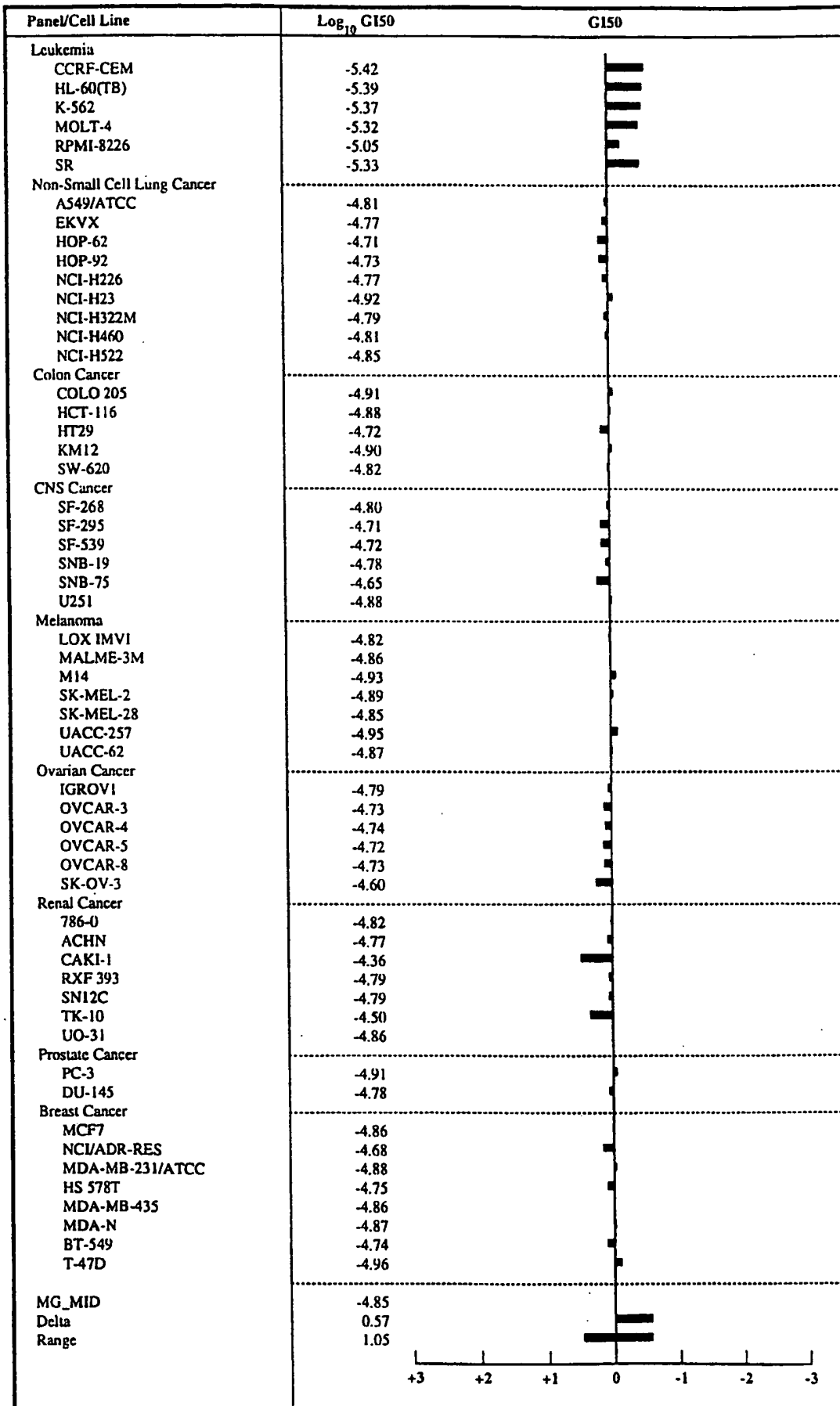
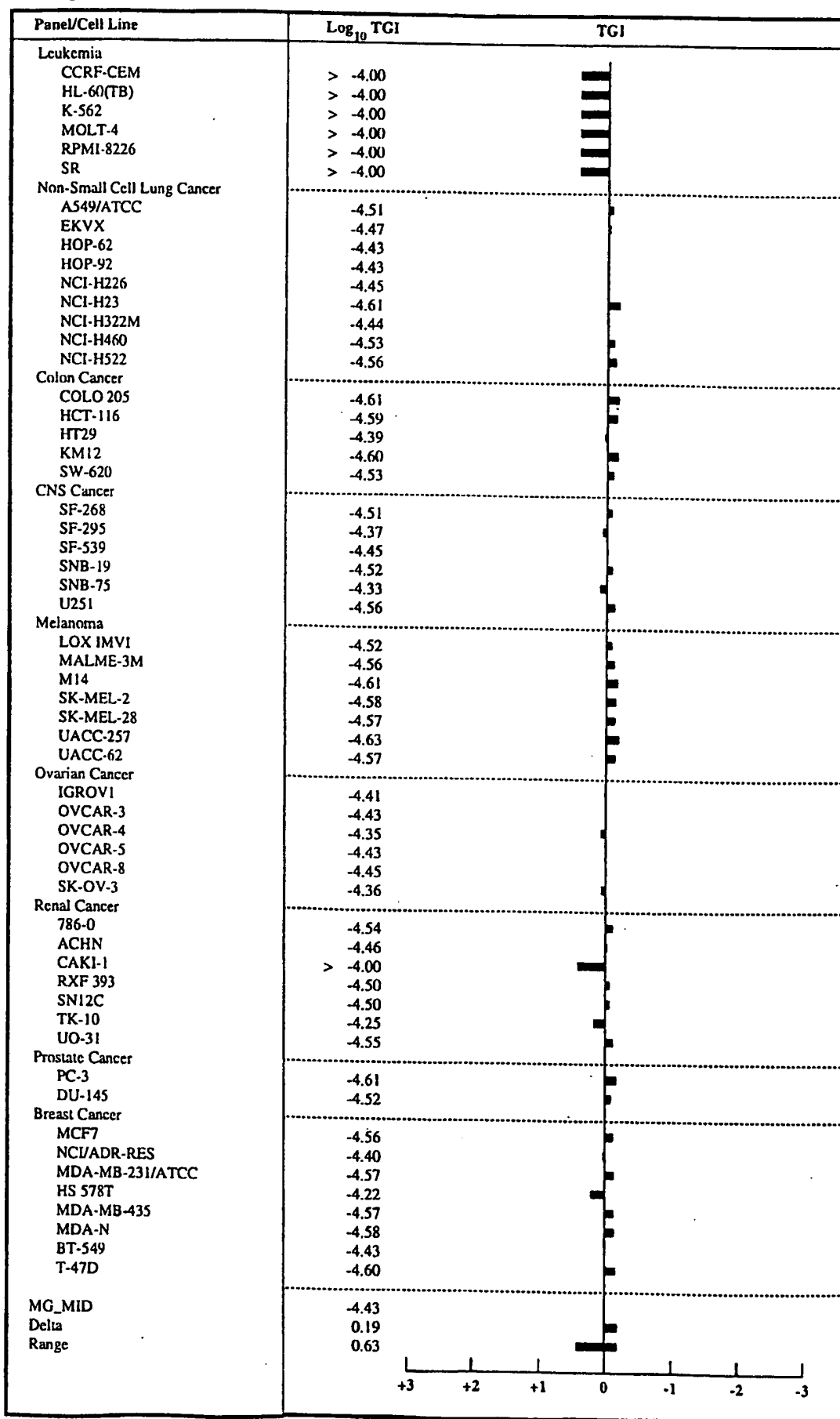


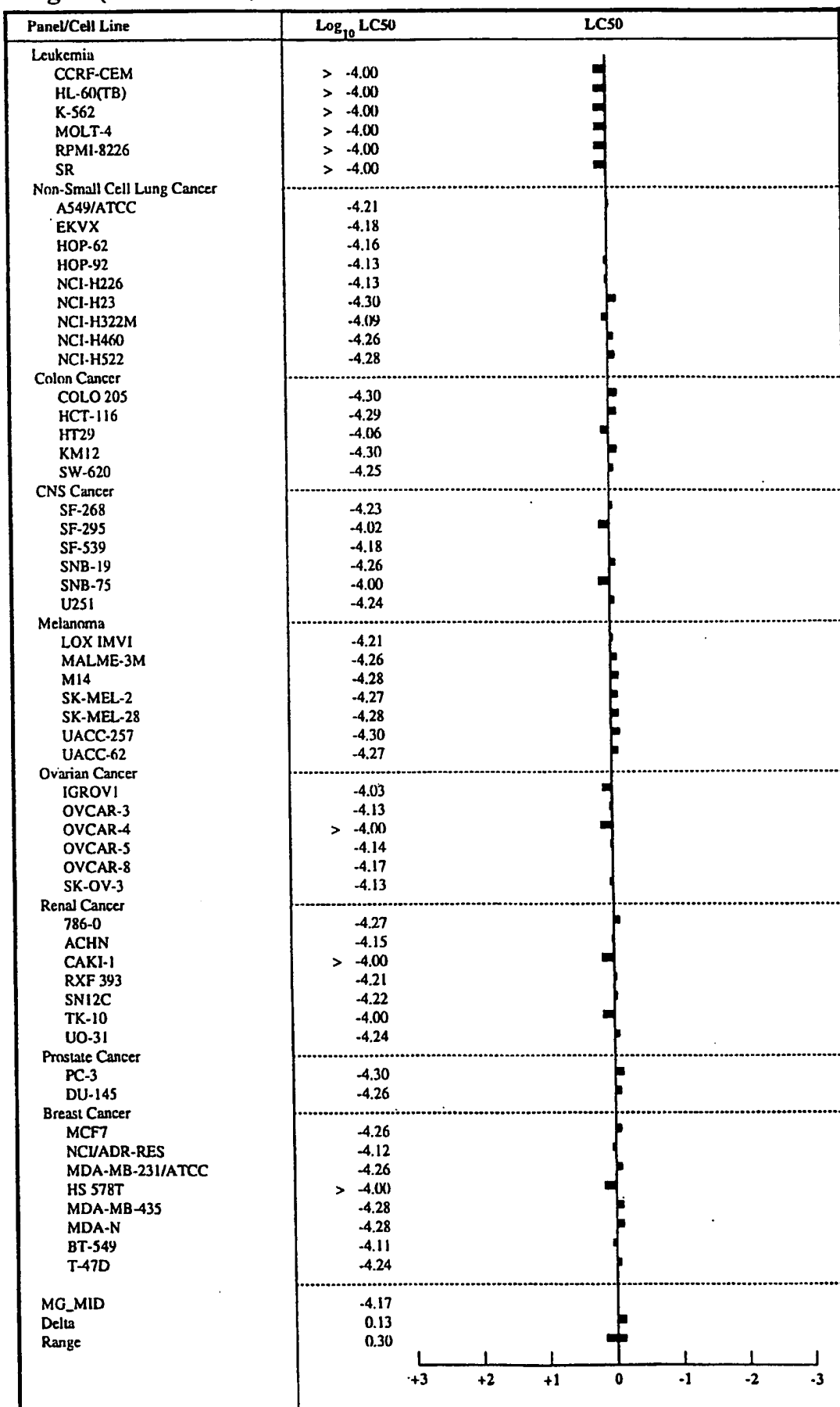
Fig. 2 (Sheet 2 of 3)

7/47



8/47

Fig. 2 (Sheet 3 of 3)



9/47

Fig. 3 (A)

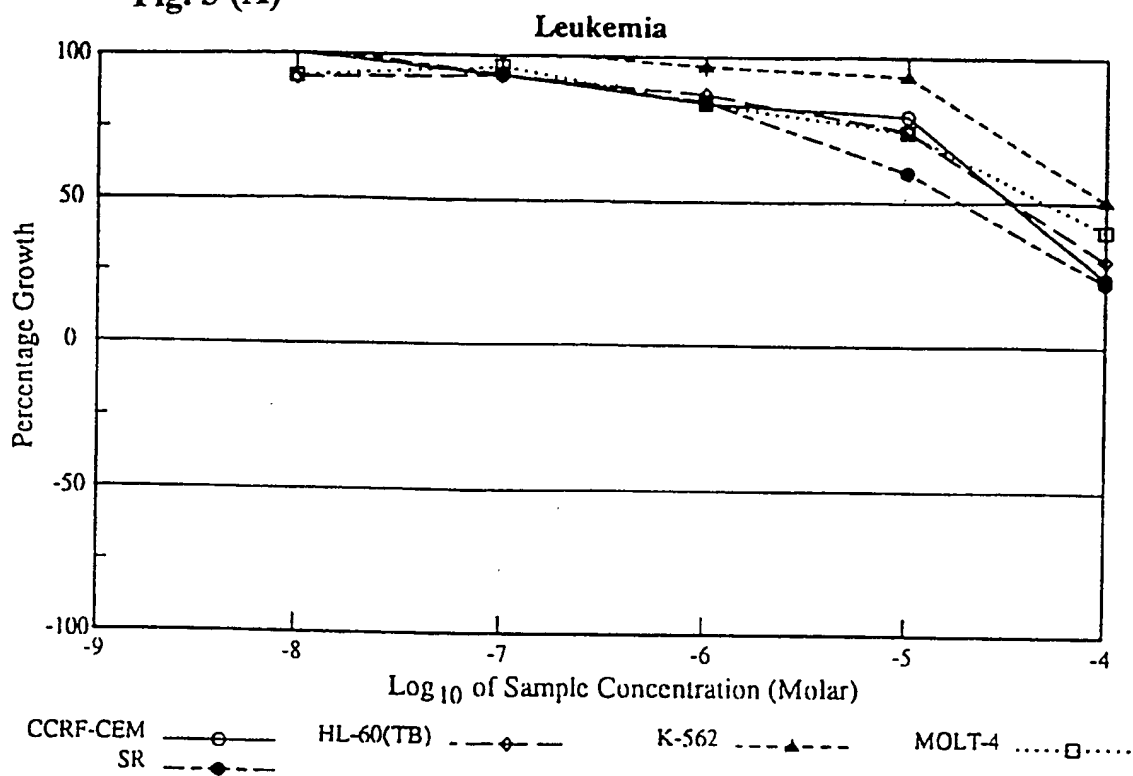
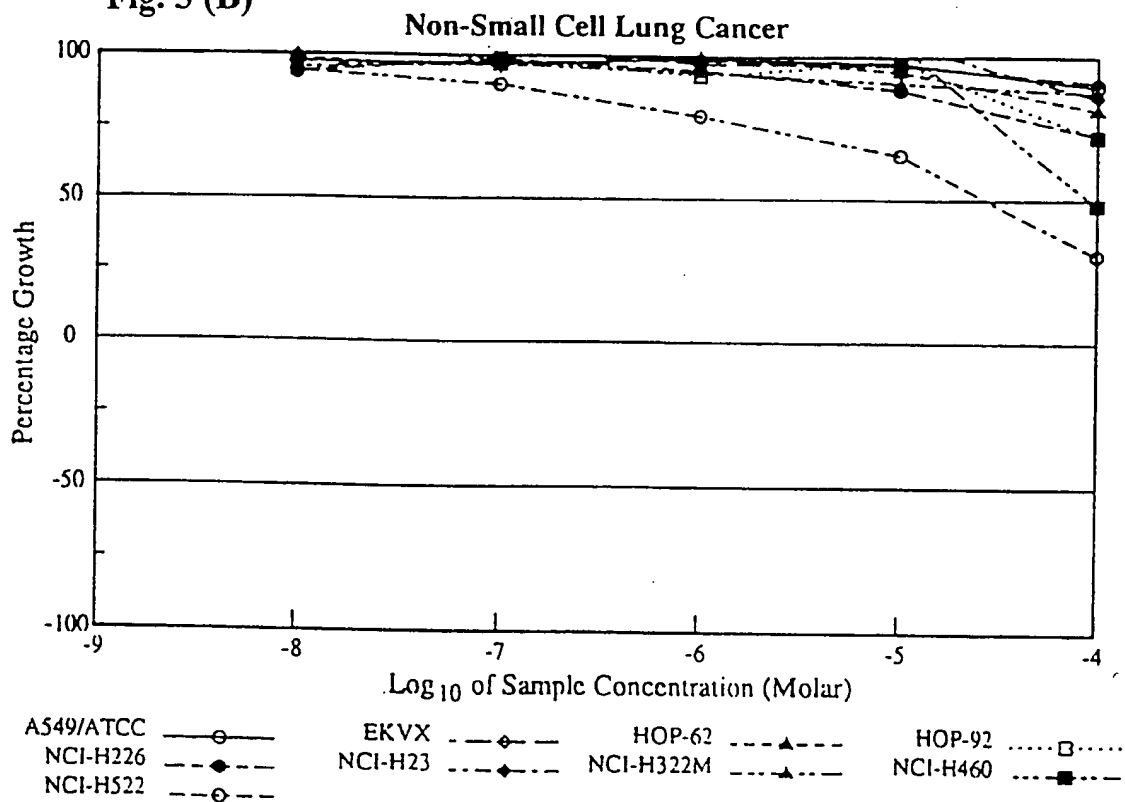
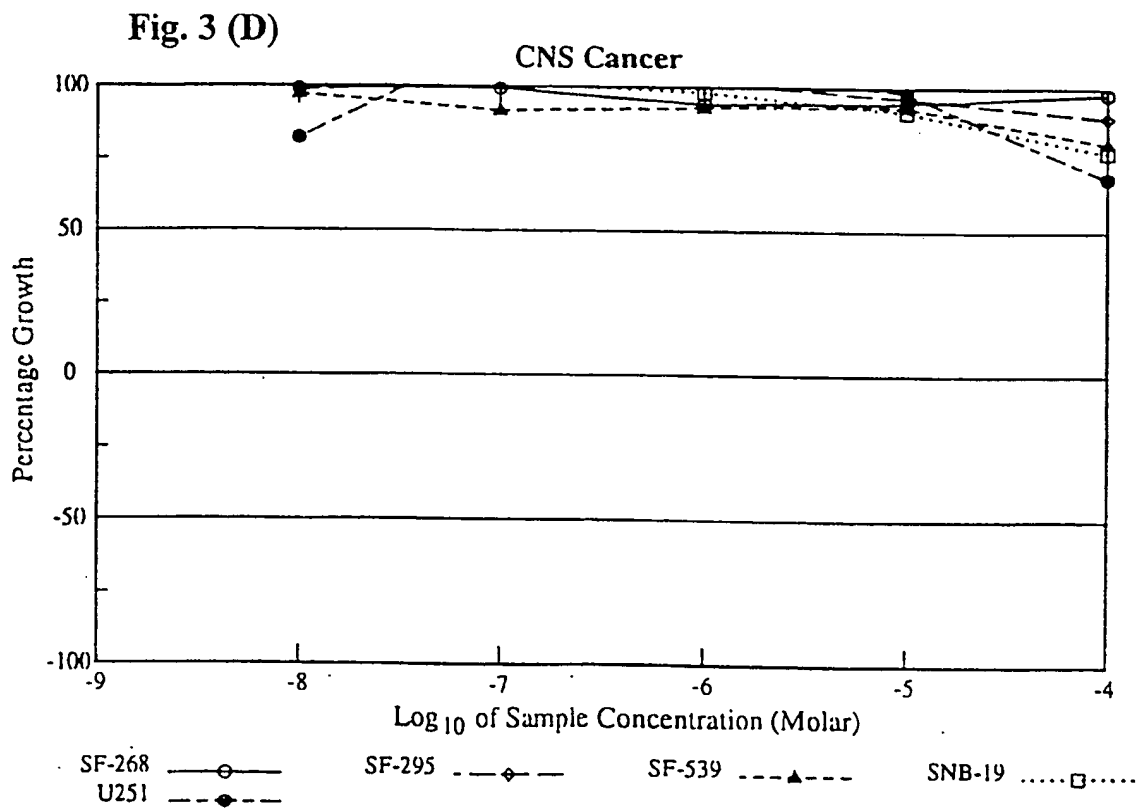
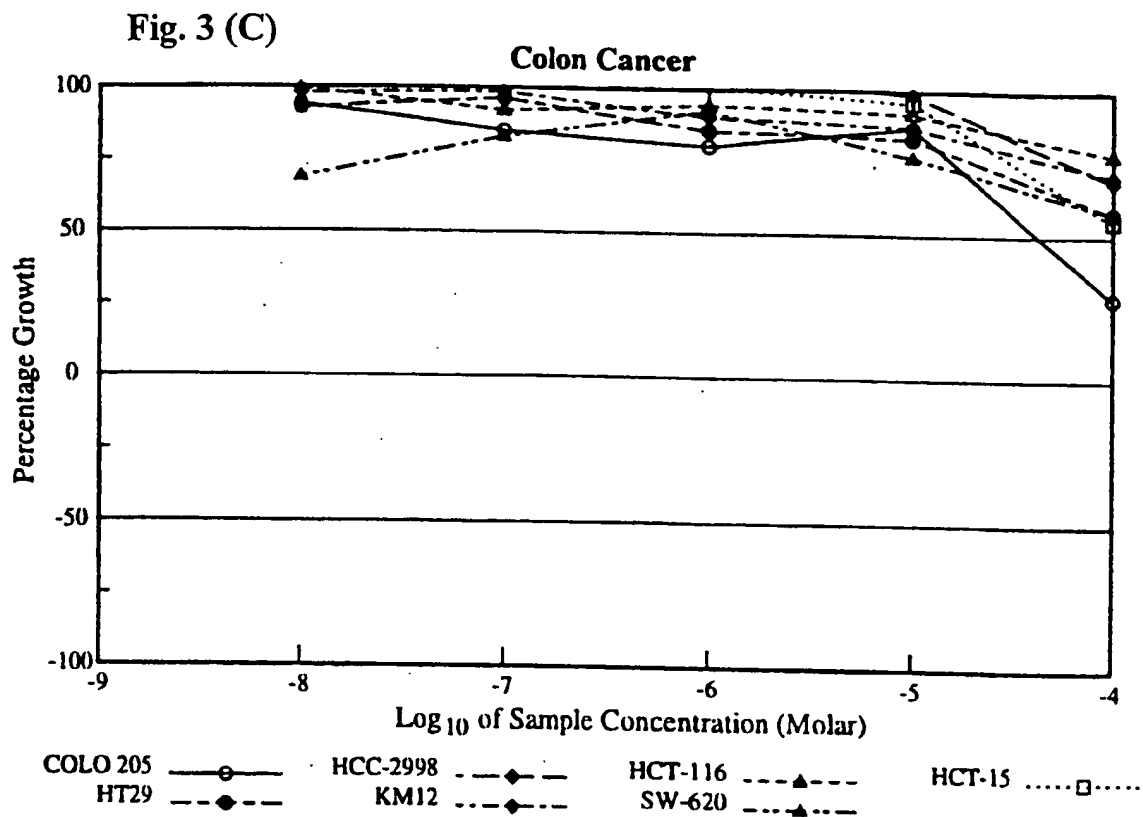


Fig. 3 (B)



10/47



11/47

Fig. 3 (E)

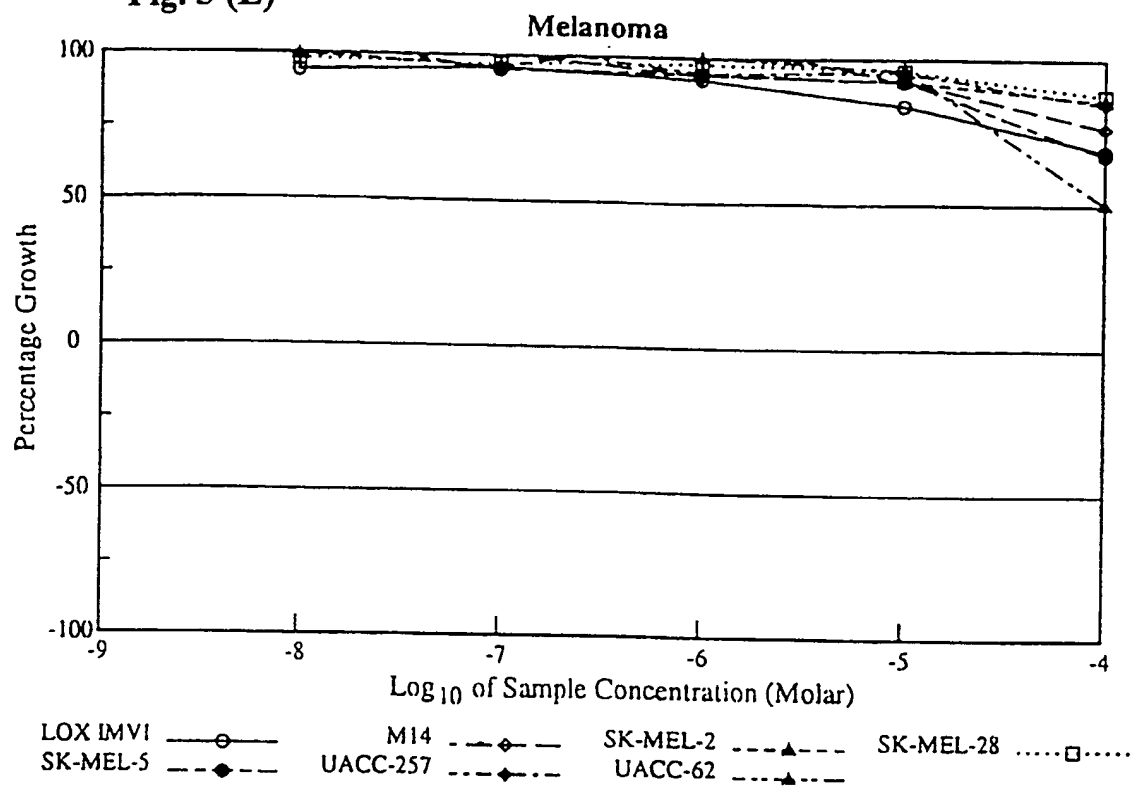
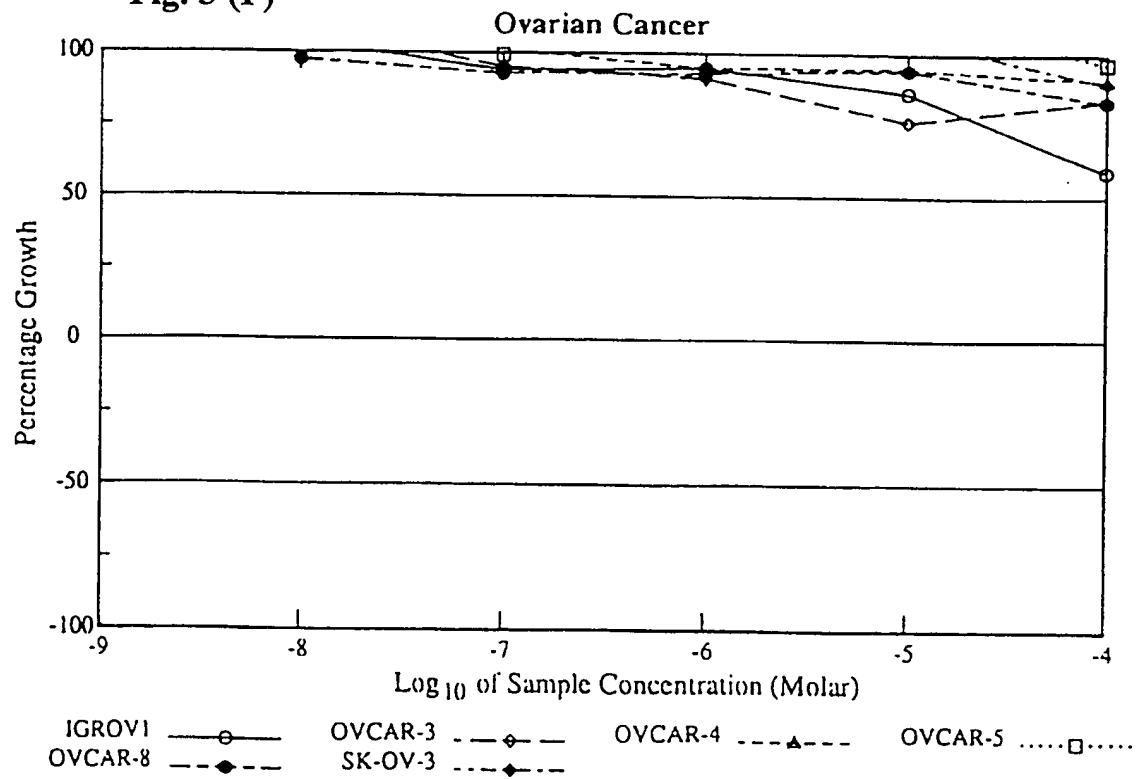


Fig. 3 (F)



12/47

Fig. 3 (G)

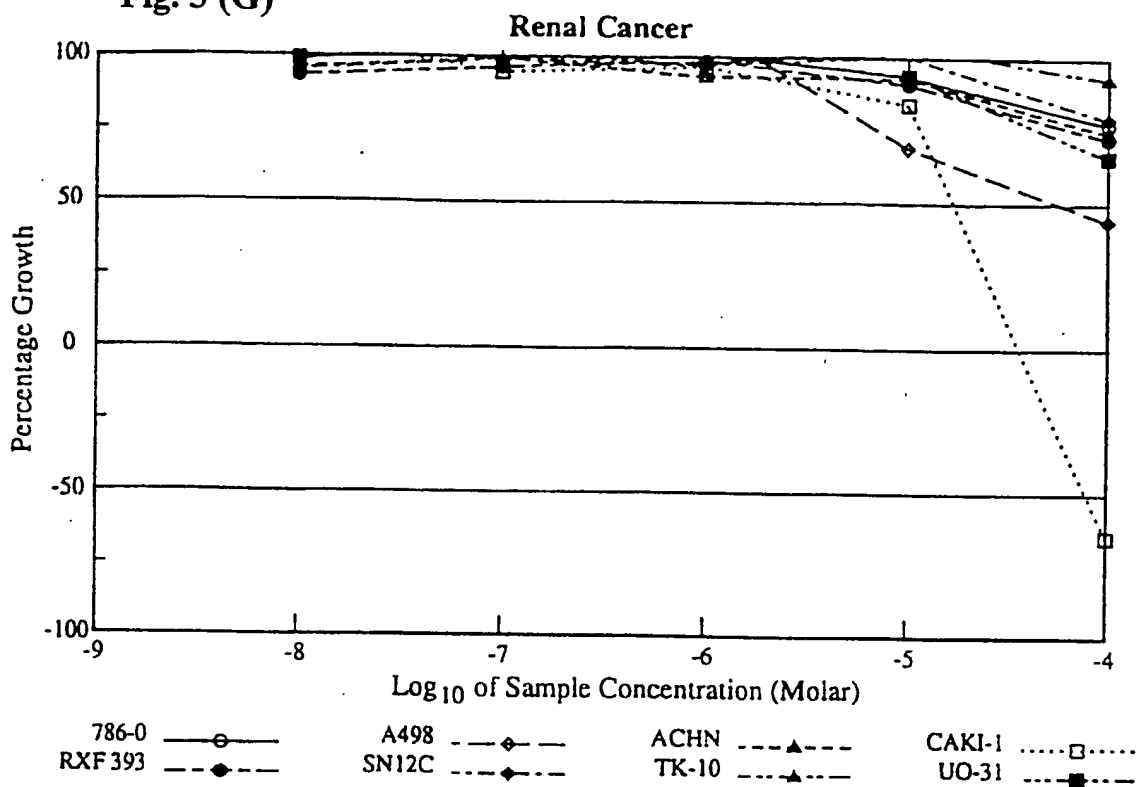
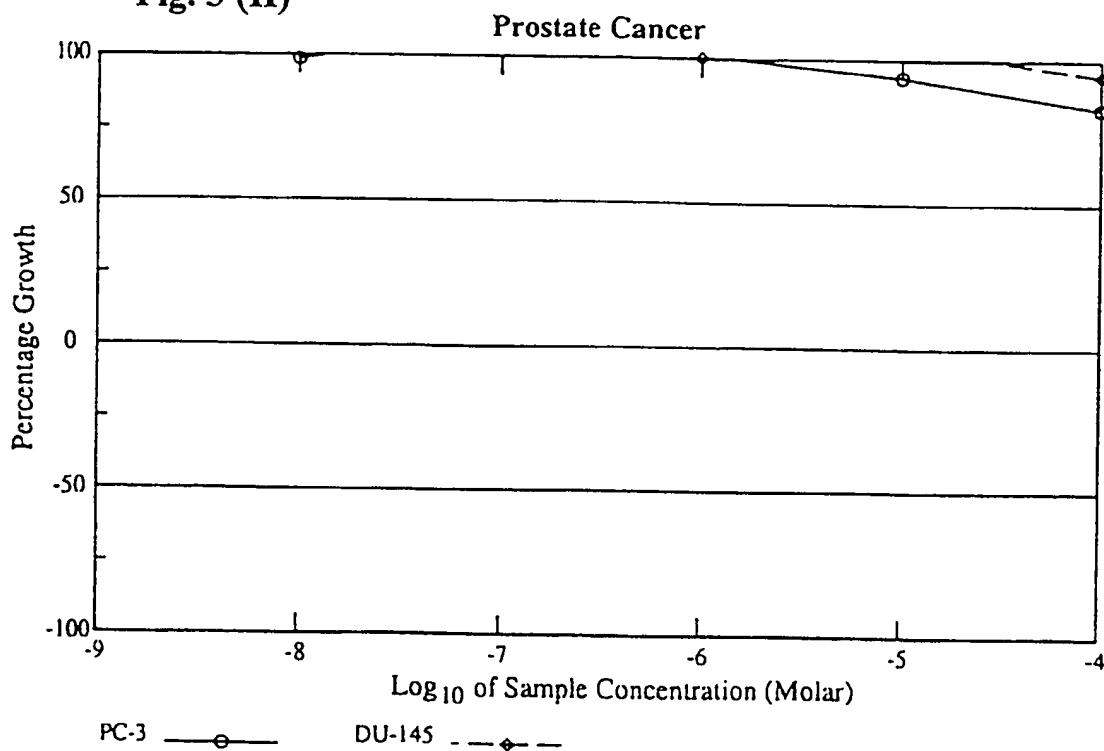


Fig. 3 (H)



13/47

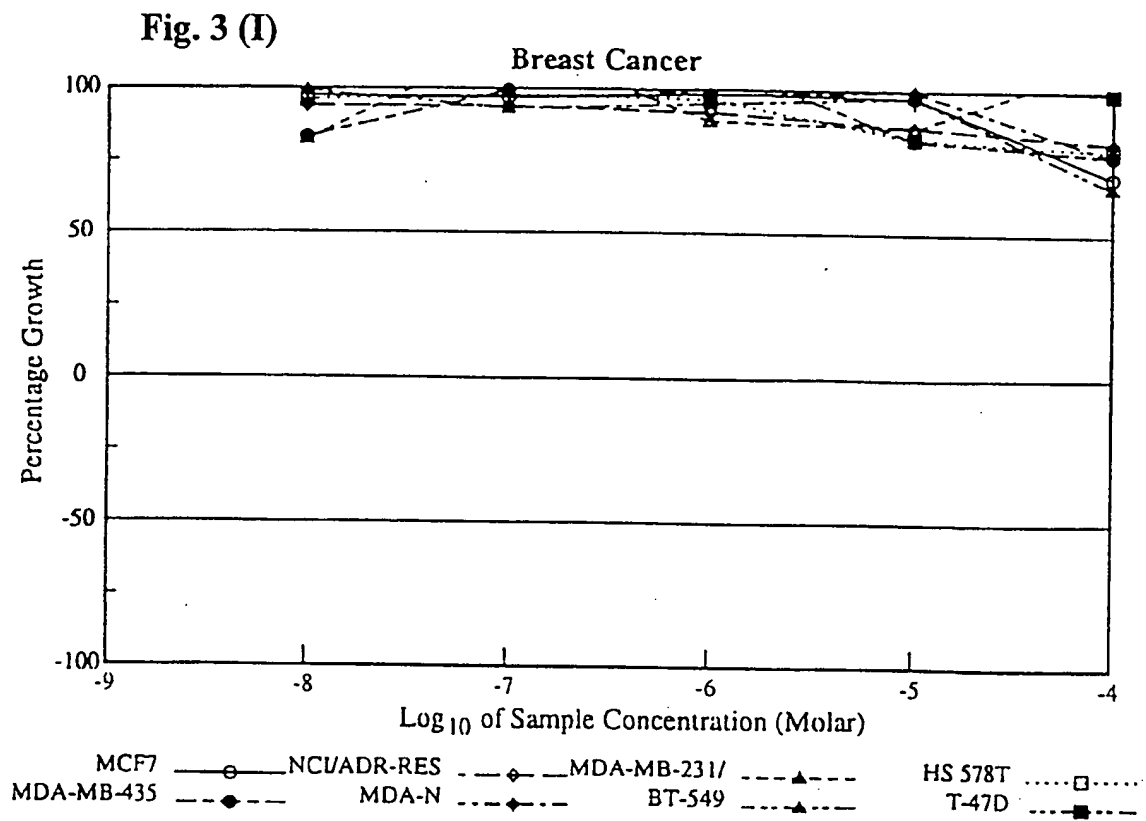


Fig. 4 (Sheet 1 of 3)

14/47

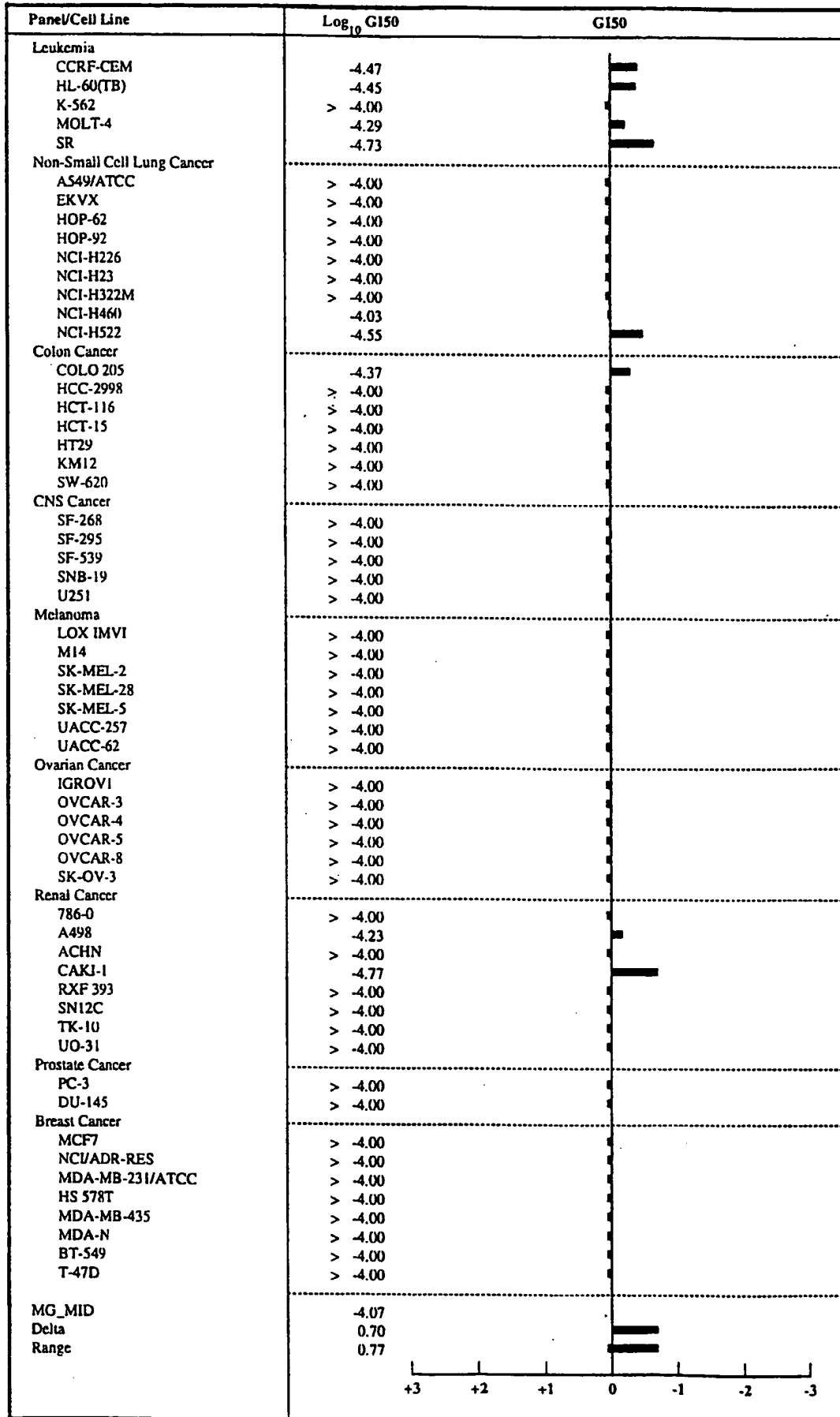


Fig. 4 (Sheet 2 of 3)

15/47

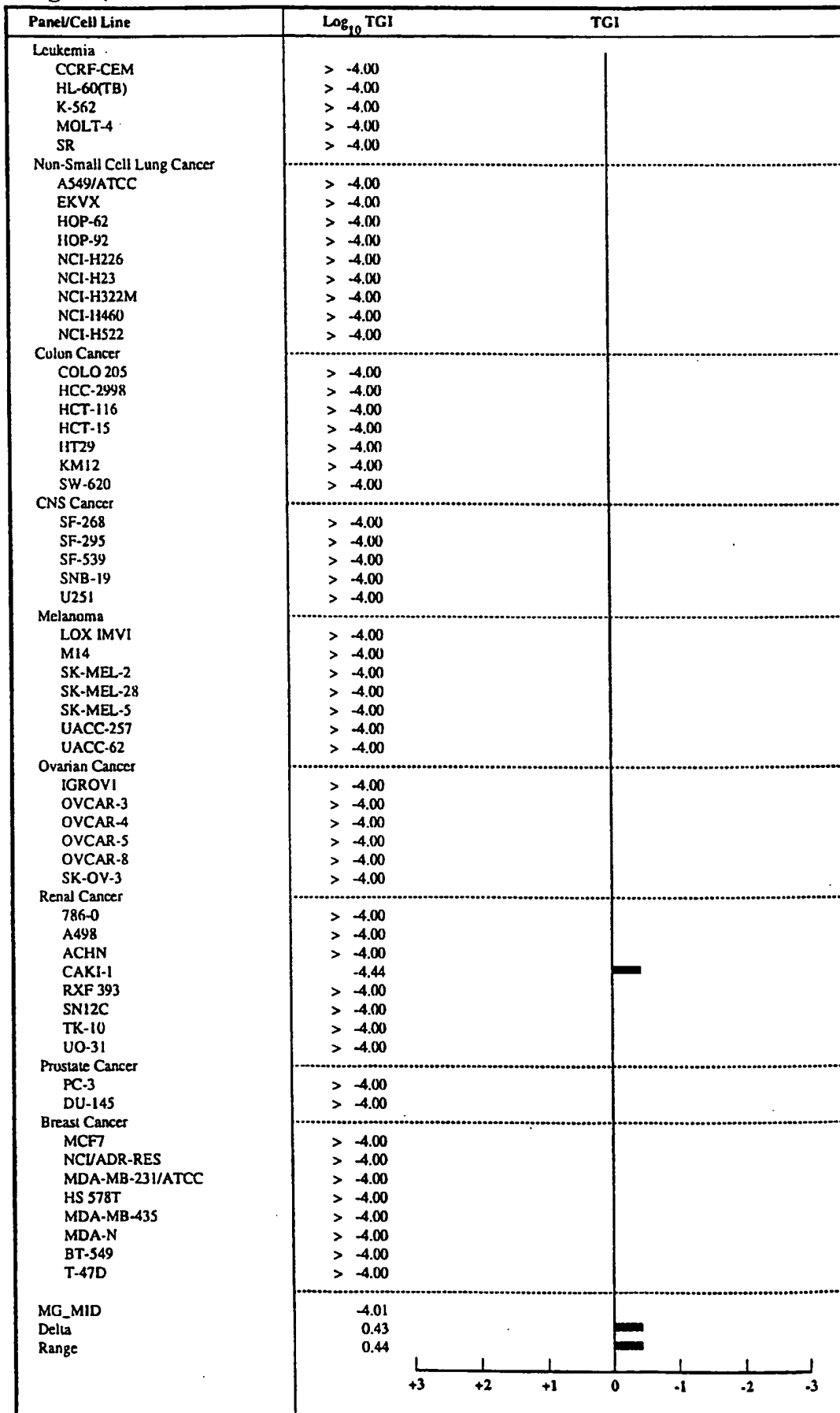
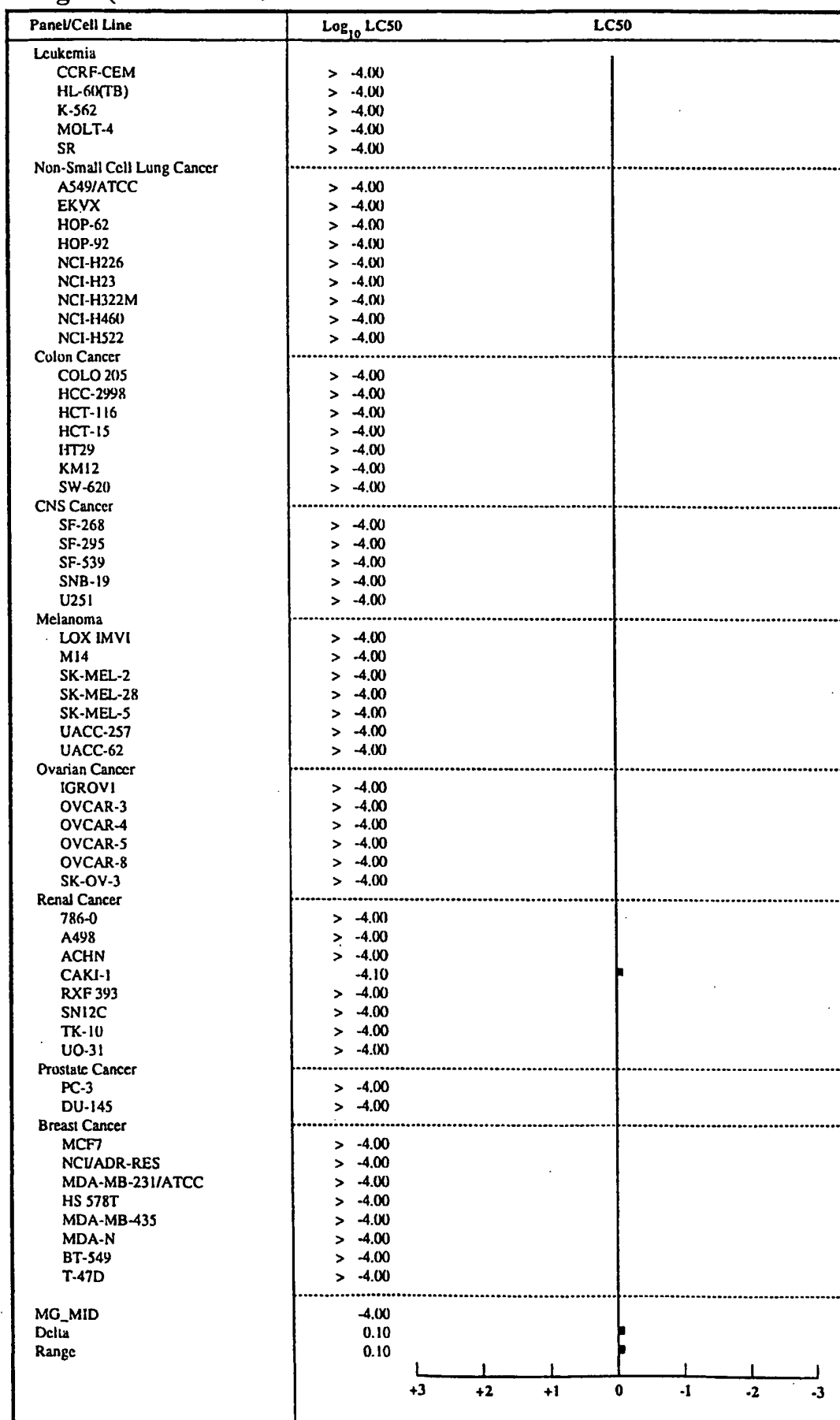


Fig. 4 (Sheet 3 of 3)

16/47



17/47

Fig. 5 (A)

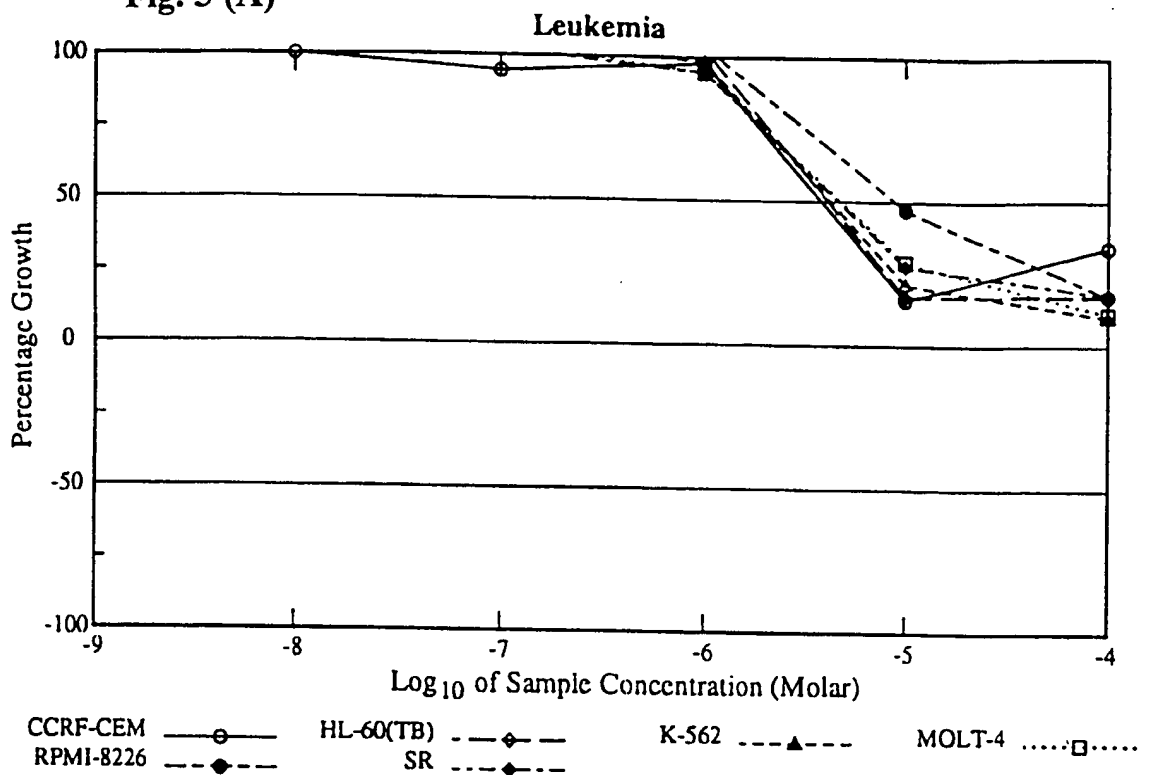
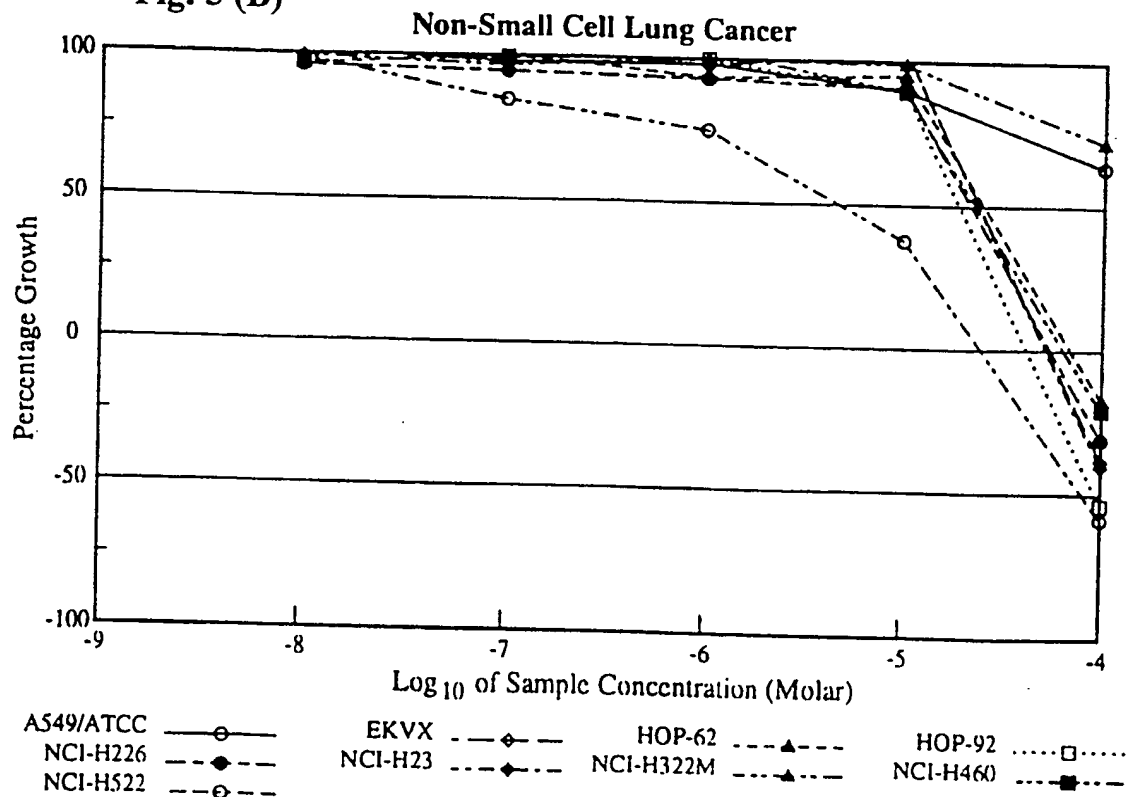


Fig. 5 (B)



18/47

Fig. 5 (C)

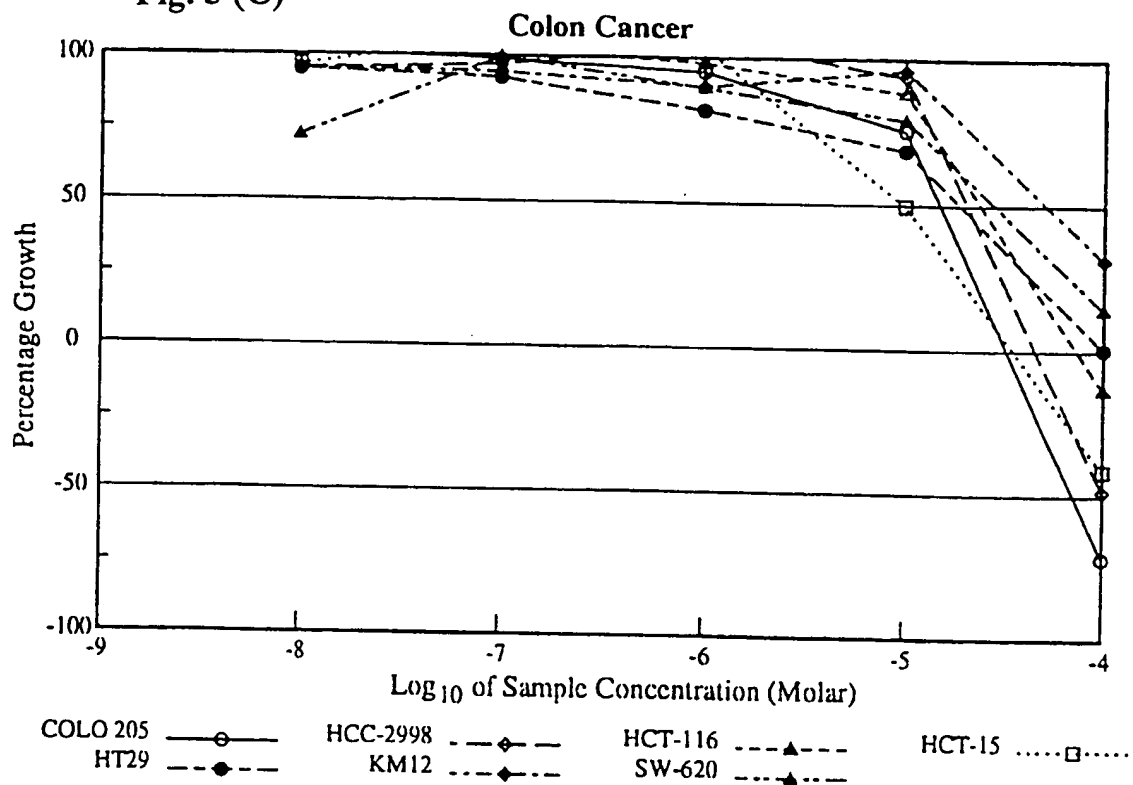
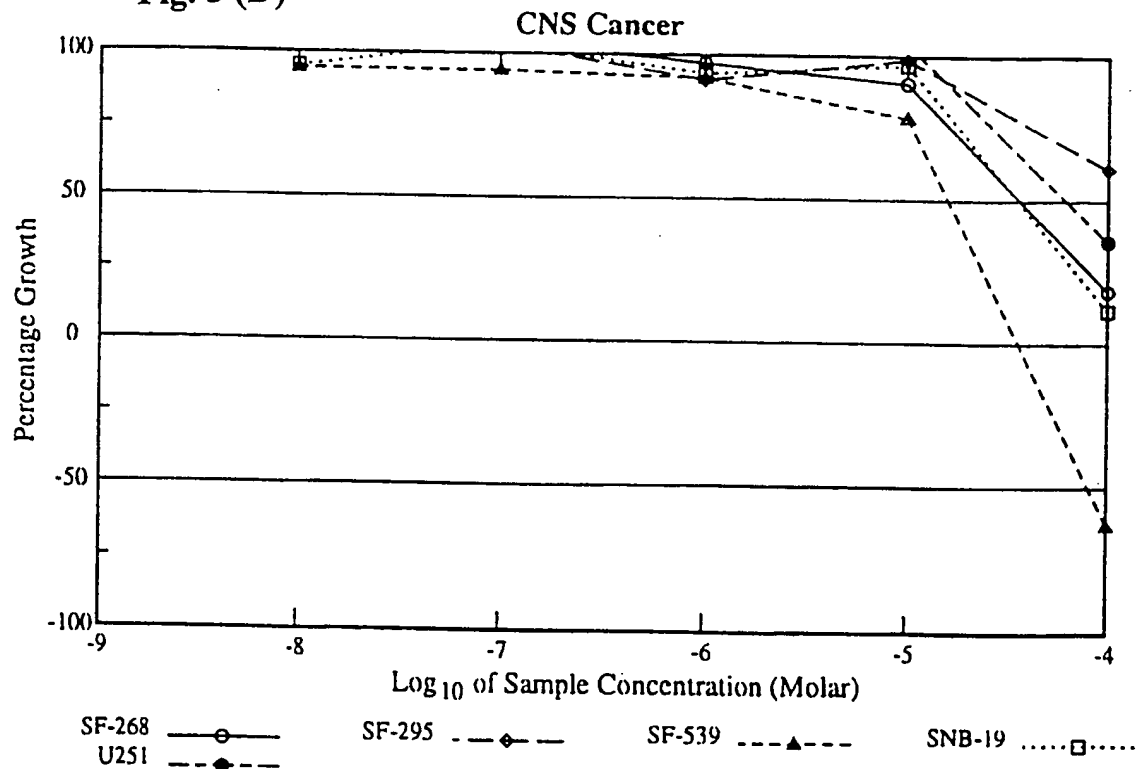


Fig. 5 (D)



19/47

Fig. 5 (E)

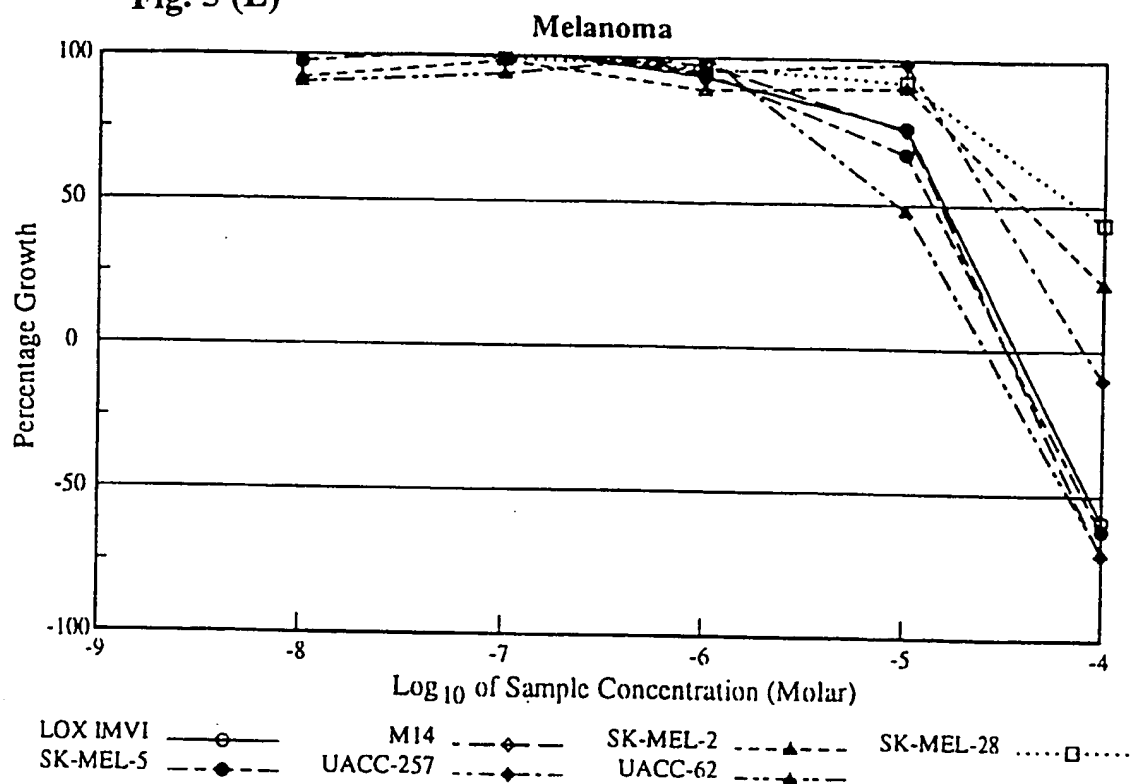
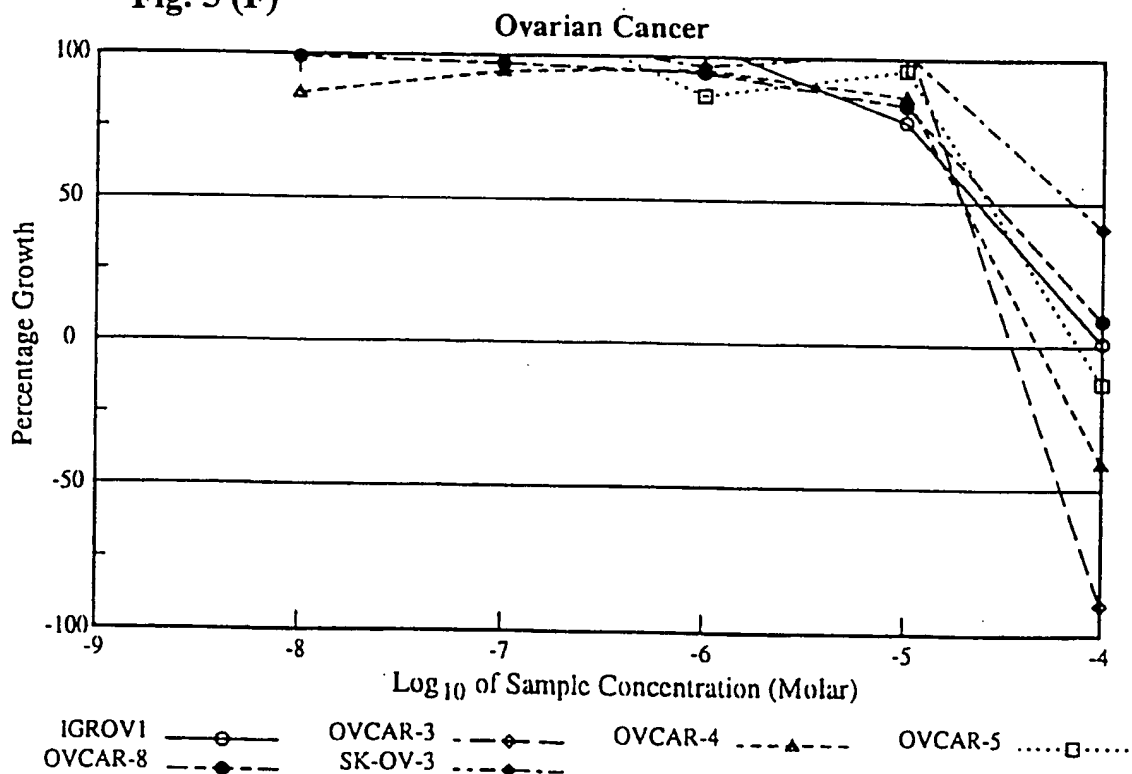


Fig. 5 (F)



20/47

Fig. 5 (G)

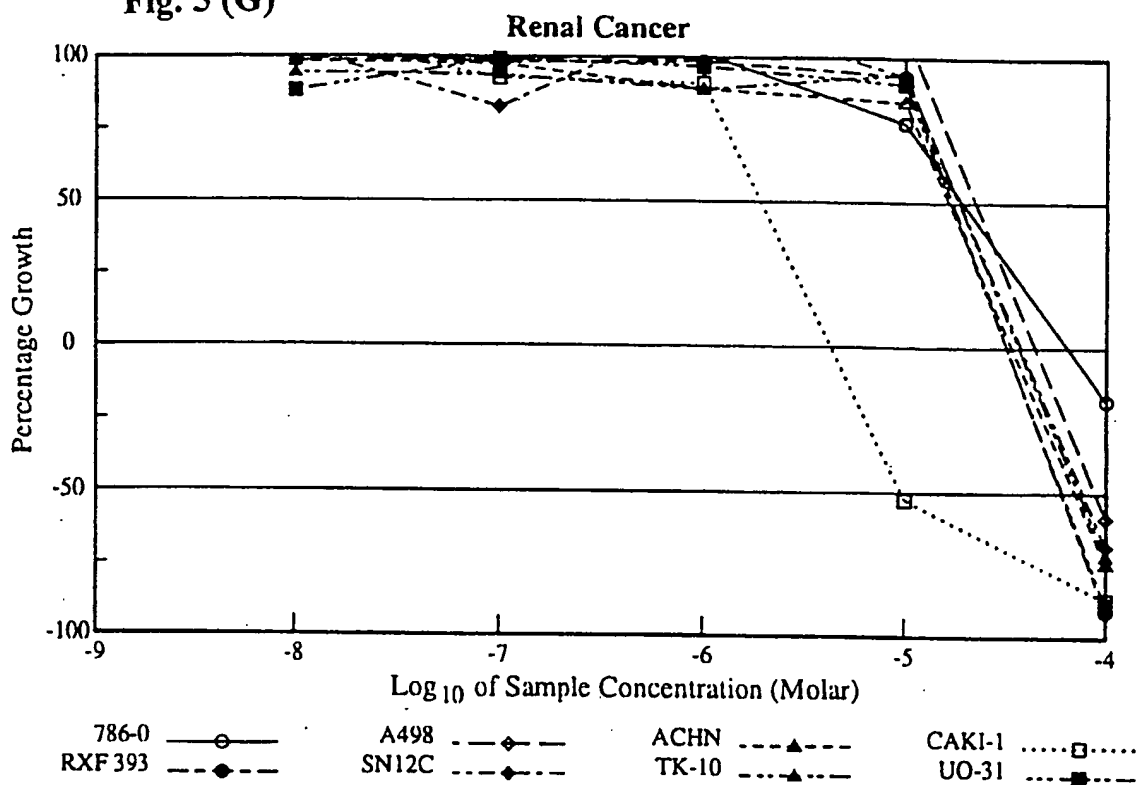
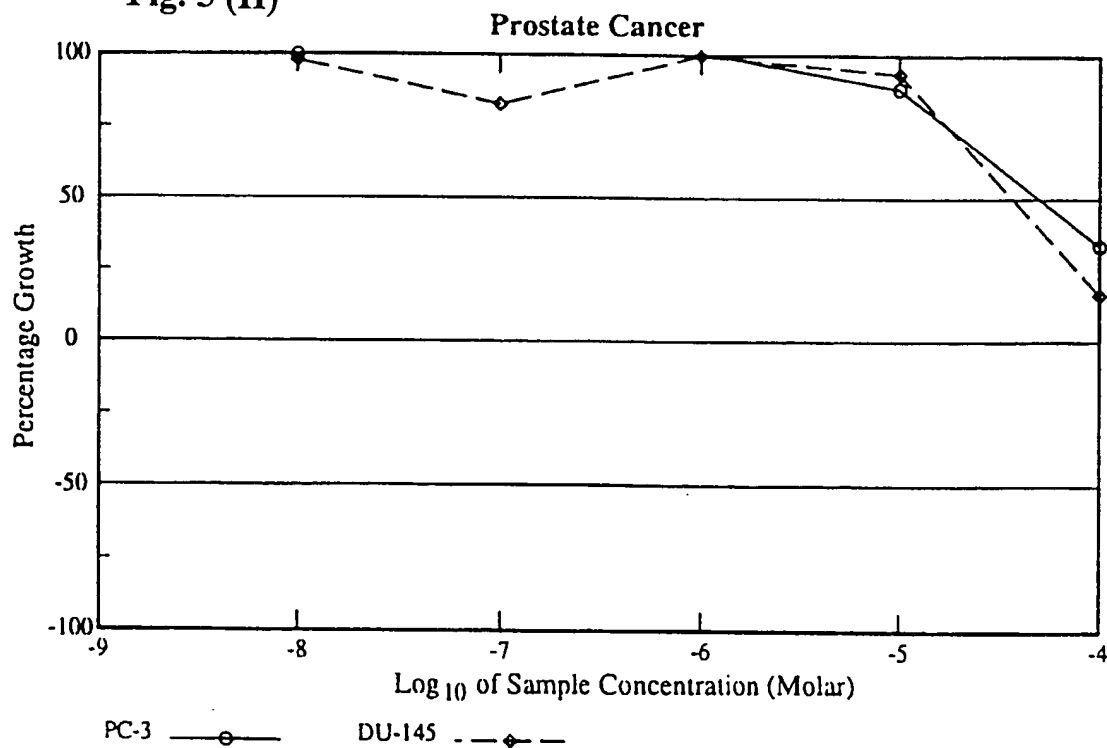


Fig. 5 (H)



21/47

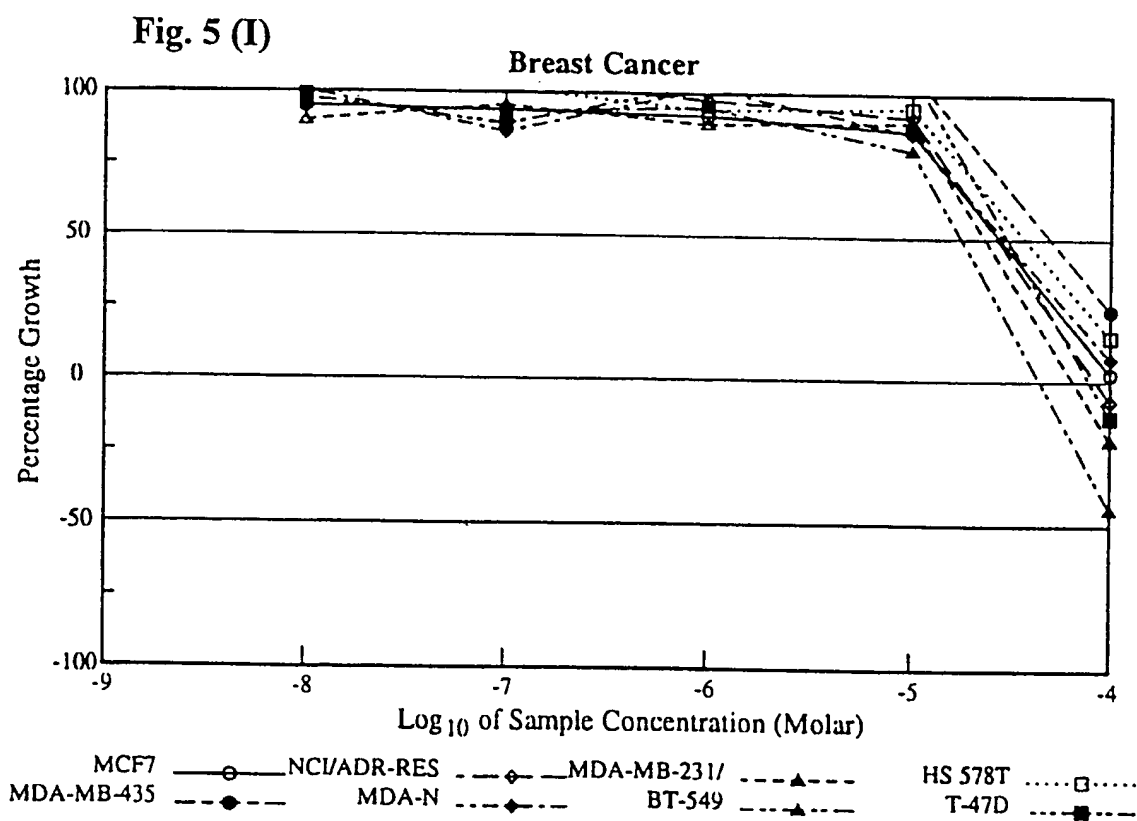


Fig. 6 (Sheet 1 of 3)

22/47

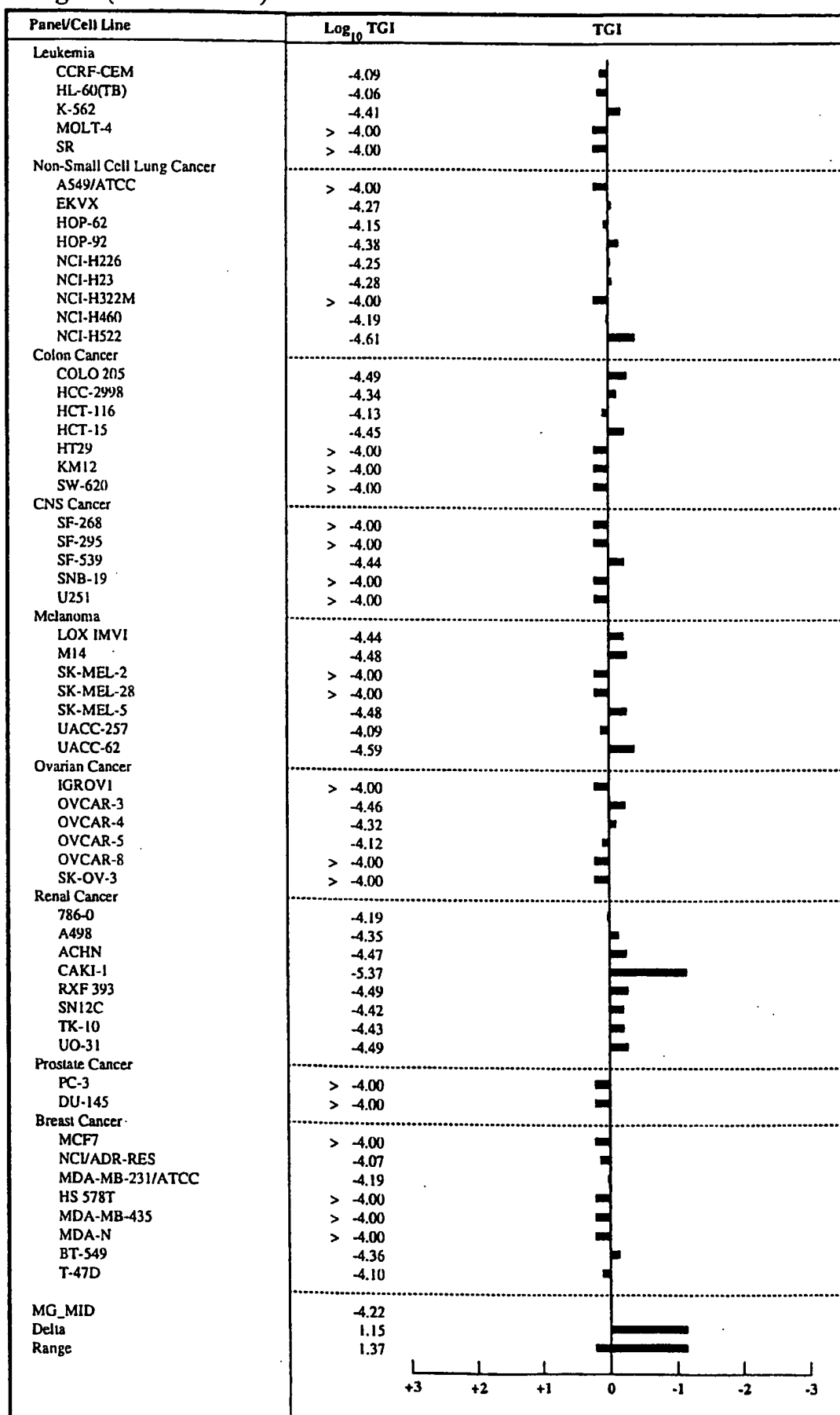


Fig. 6 (Sheet 2 of 3)

23/47

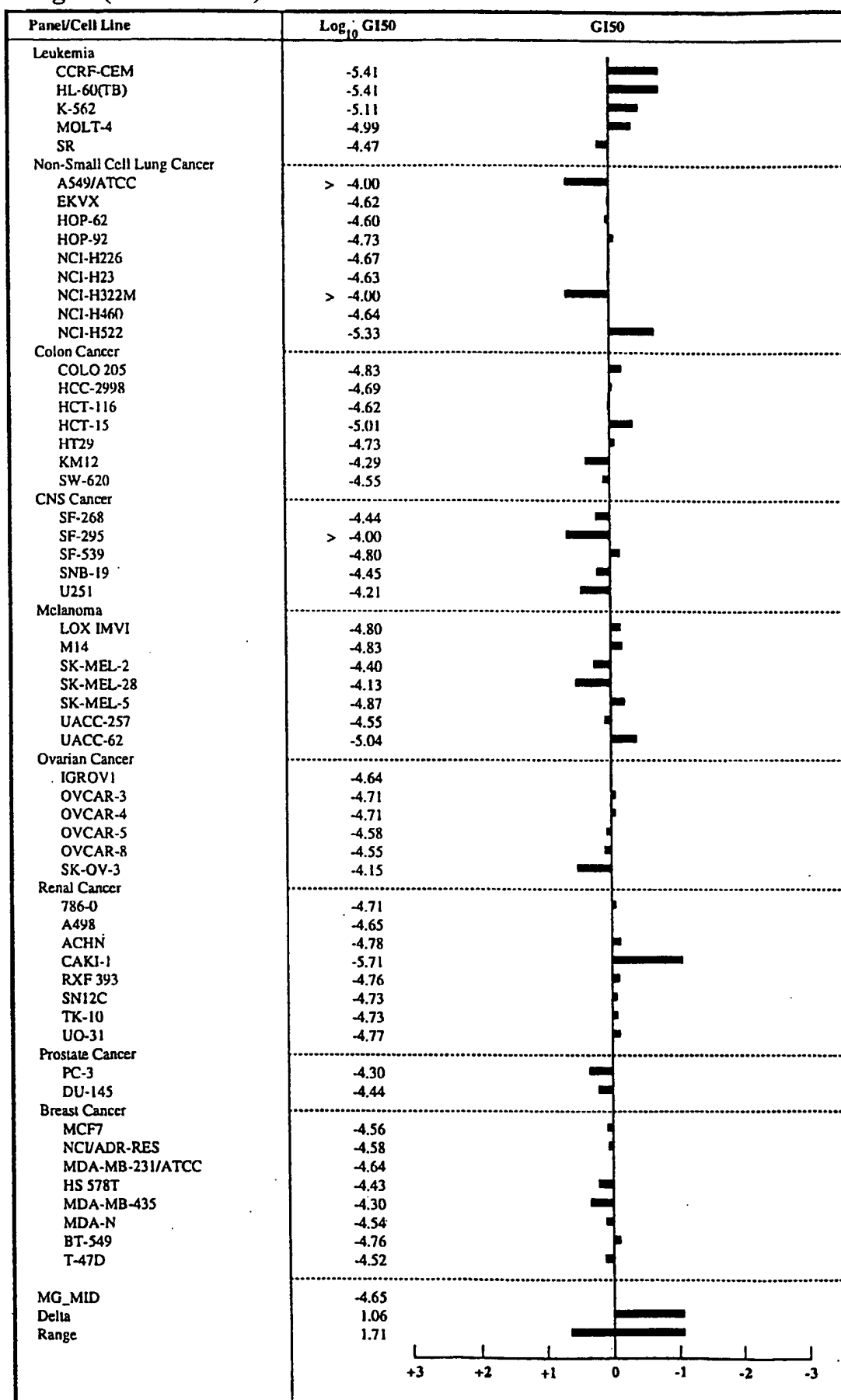
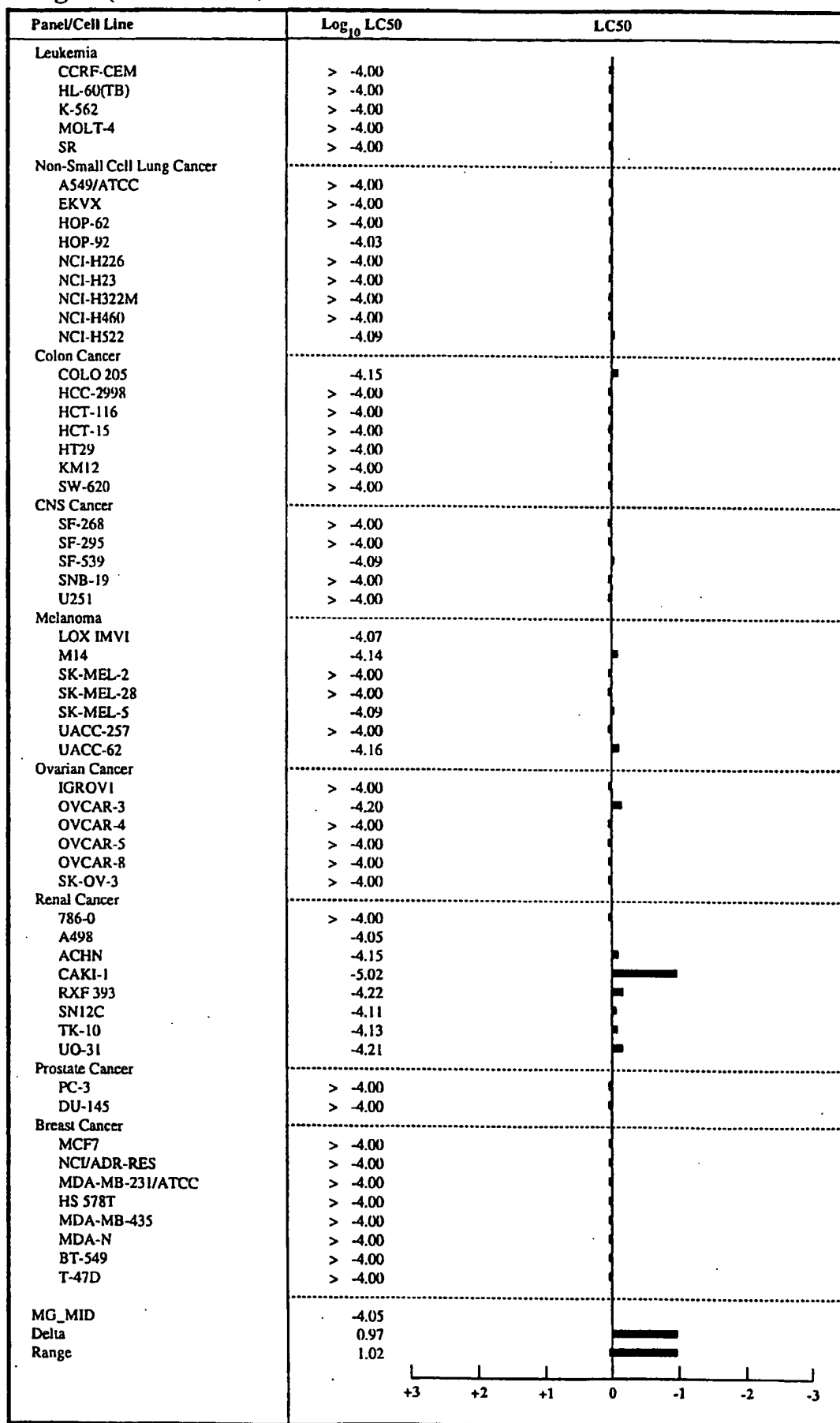


Fig. 6 (Sheet 3 of 3)

24/47



25/47

Fig. 7 (A)

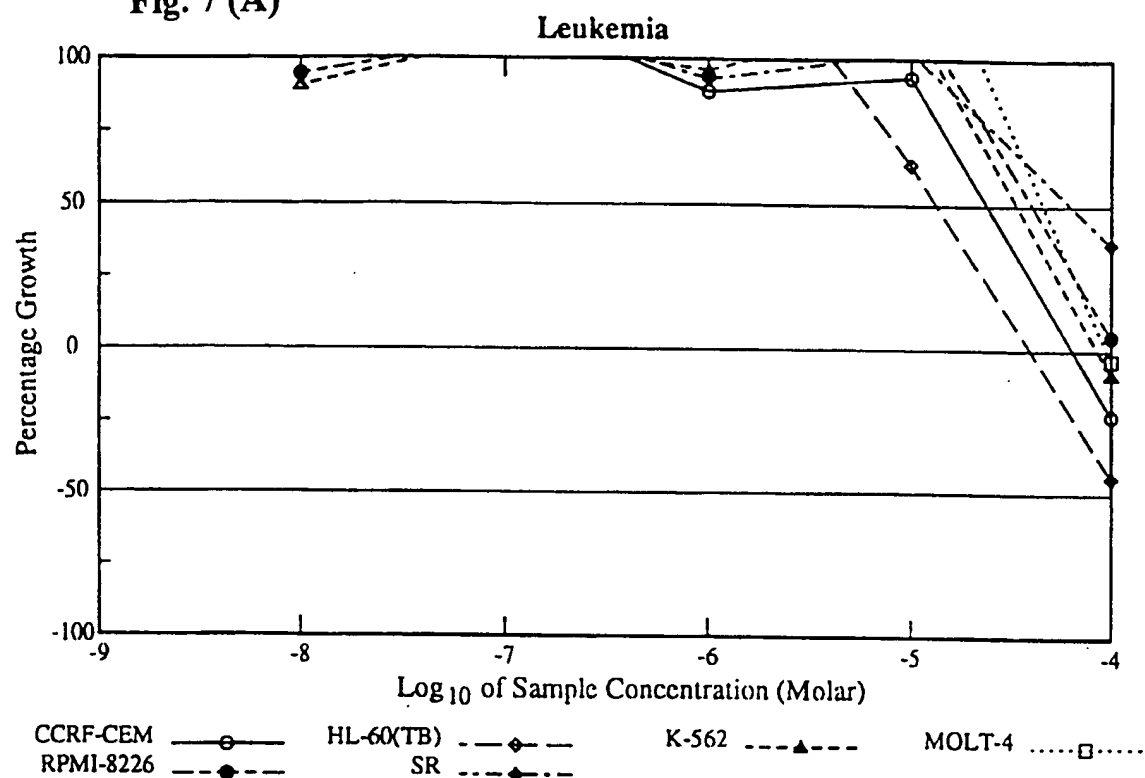
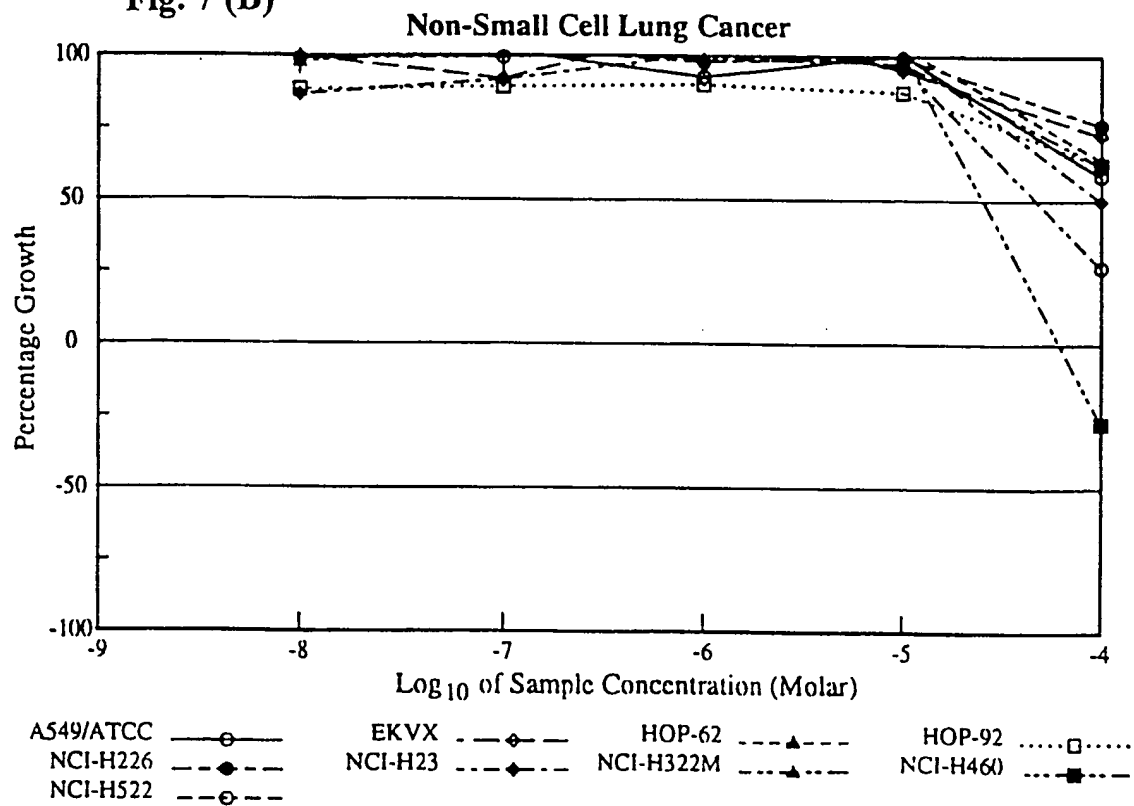


Fig. 7 (B)



26/47

Fig. 7 (C)

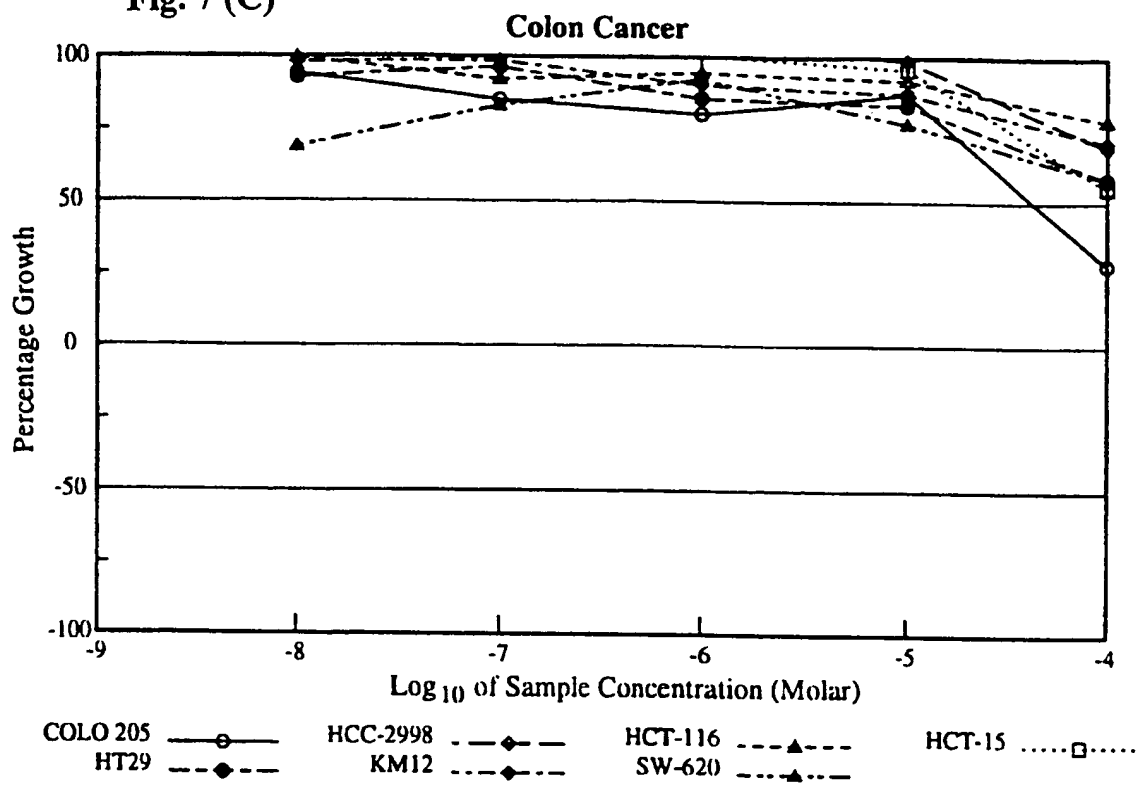
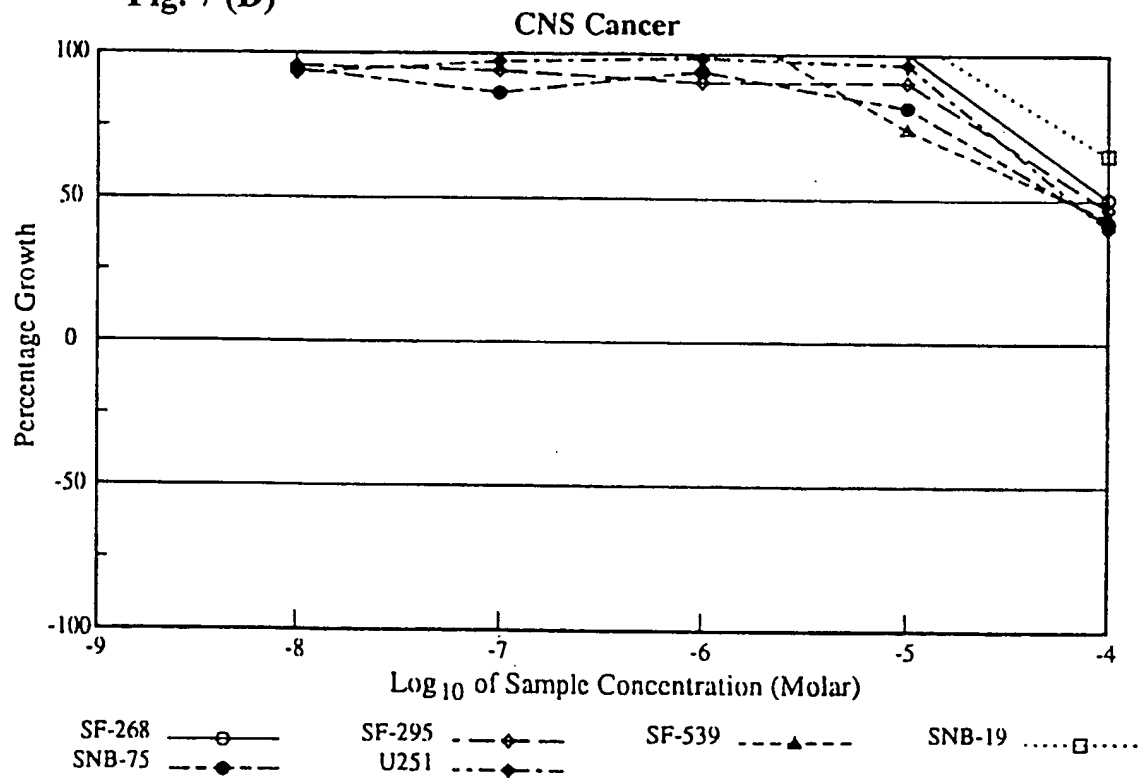


Fig. 7 (D)



27/47

Fig. 7 (E)

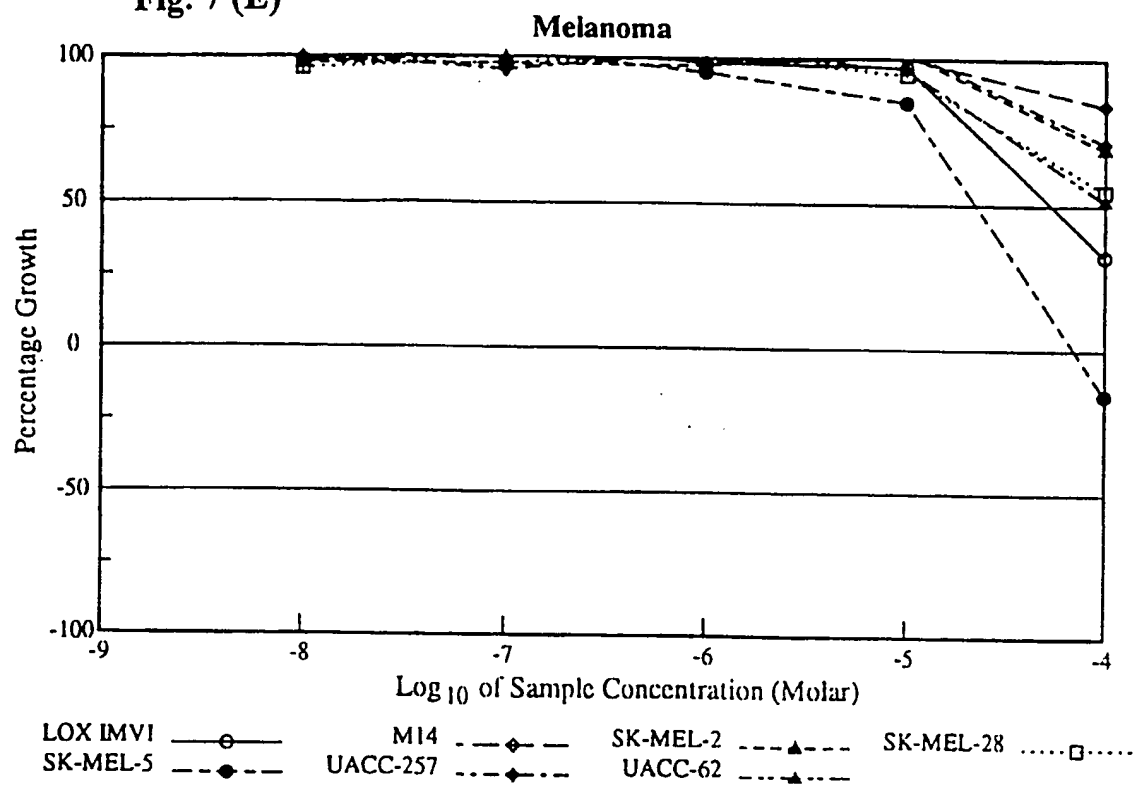
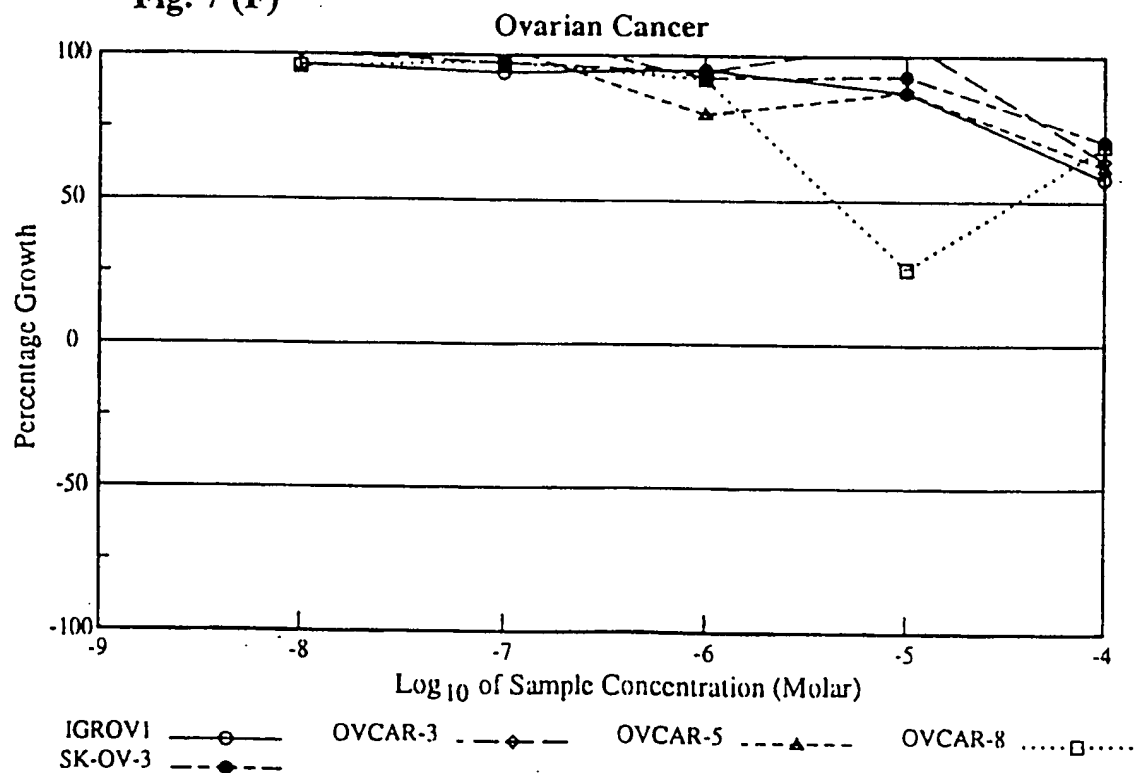


Fig. 7 (F)



28/47

Fig. 7 (G)

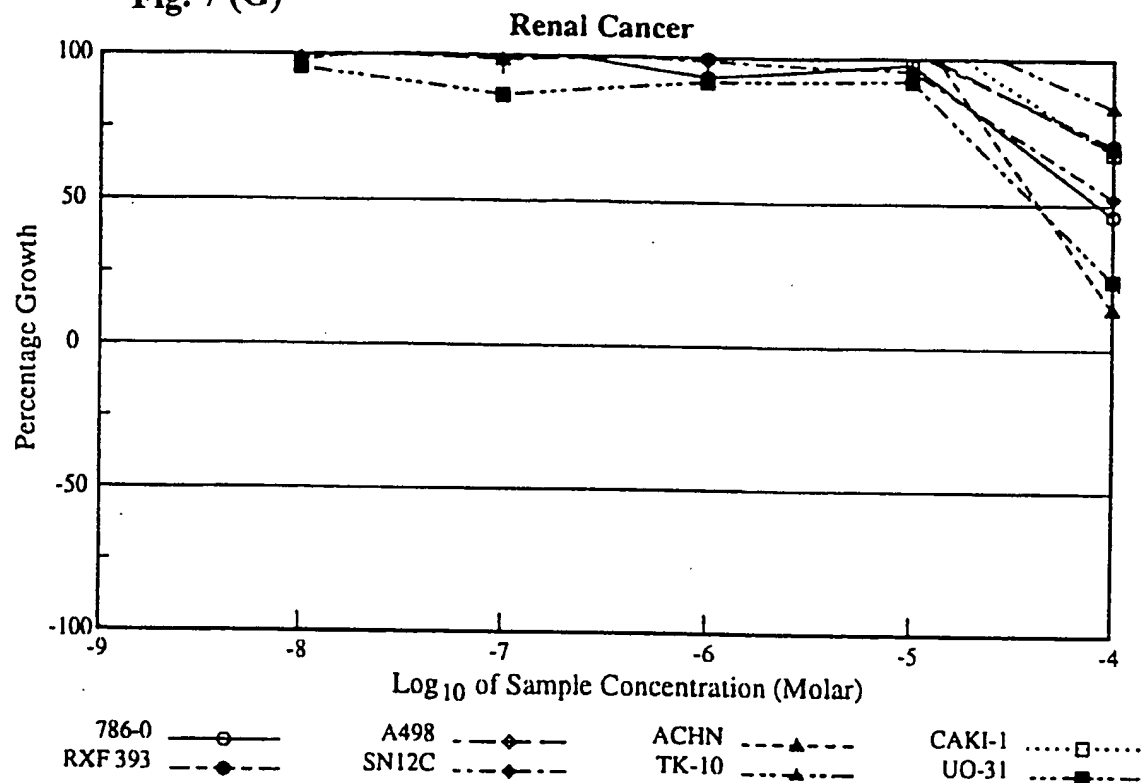
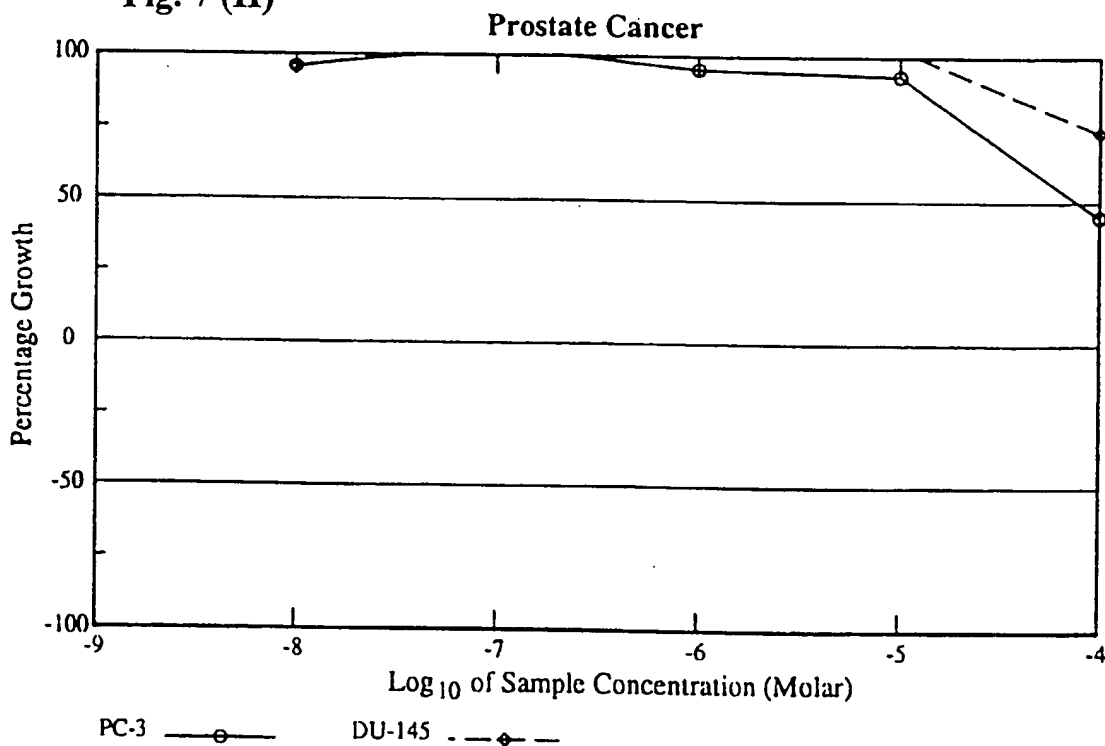


Fig. 7 (H)



29/47

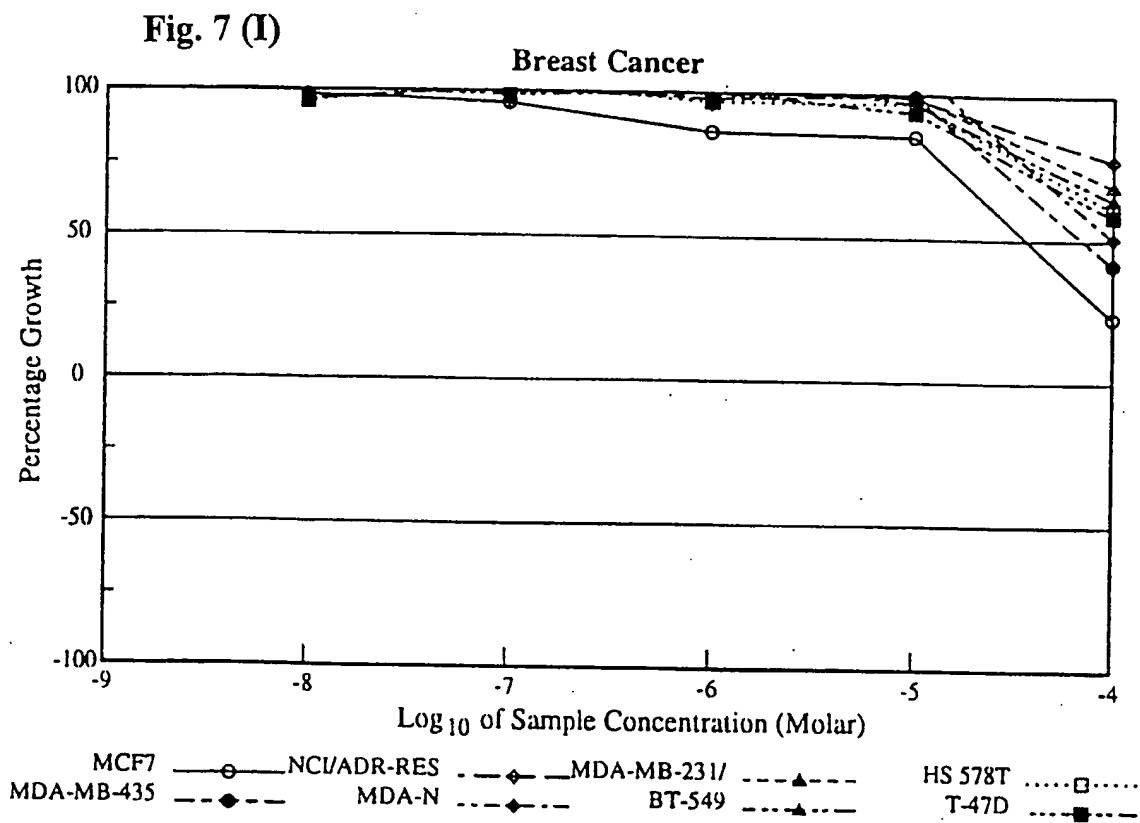


Fig. 8 (Sheet 1 of 3)

30/47

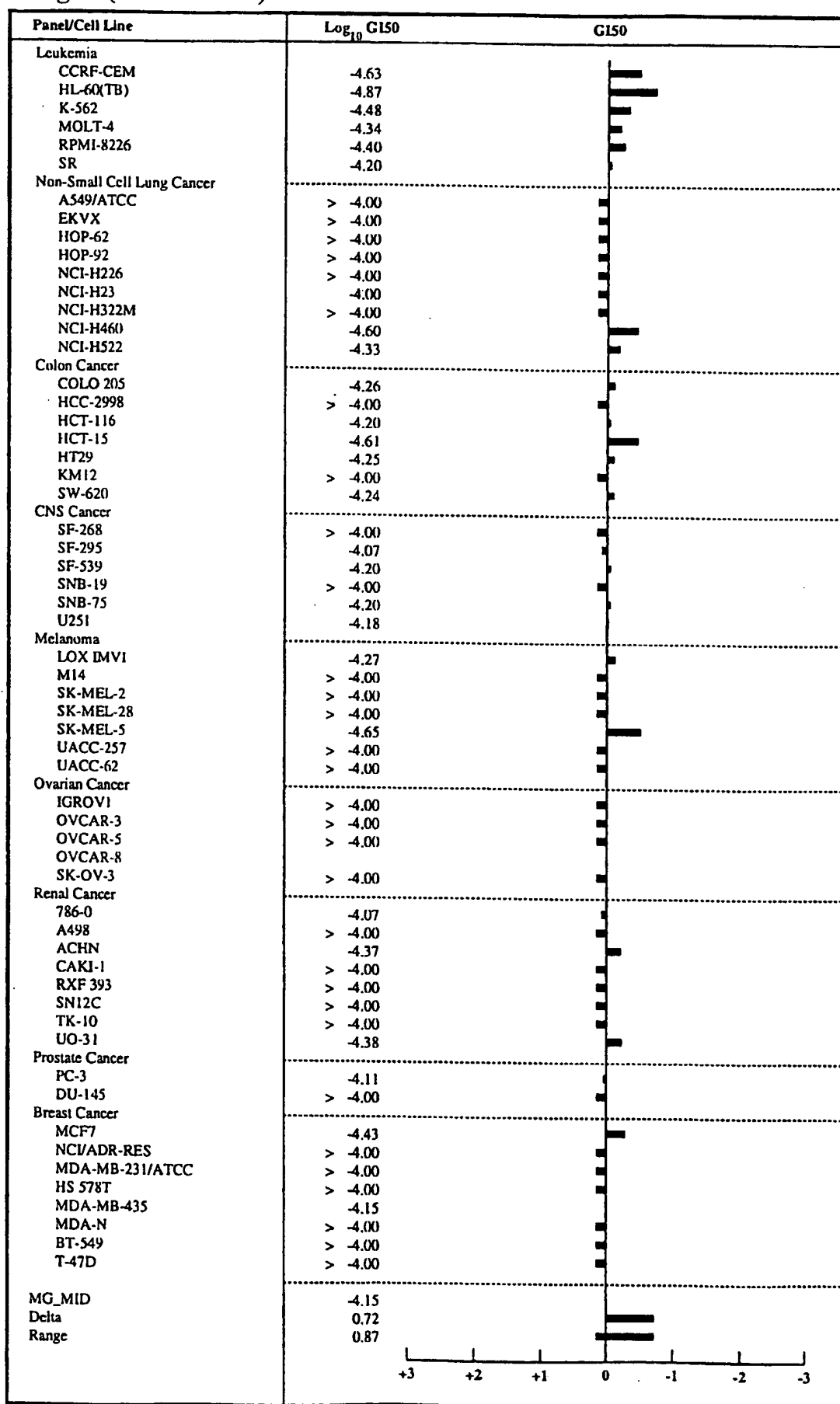


Fig. 8 (Sheet 2 of 3)

31/47

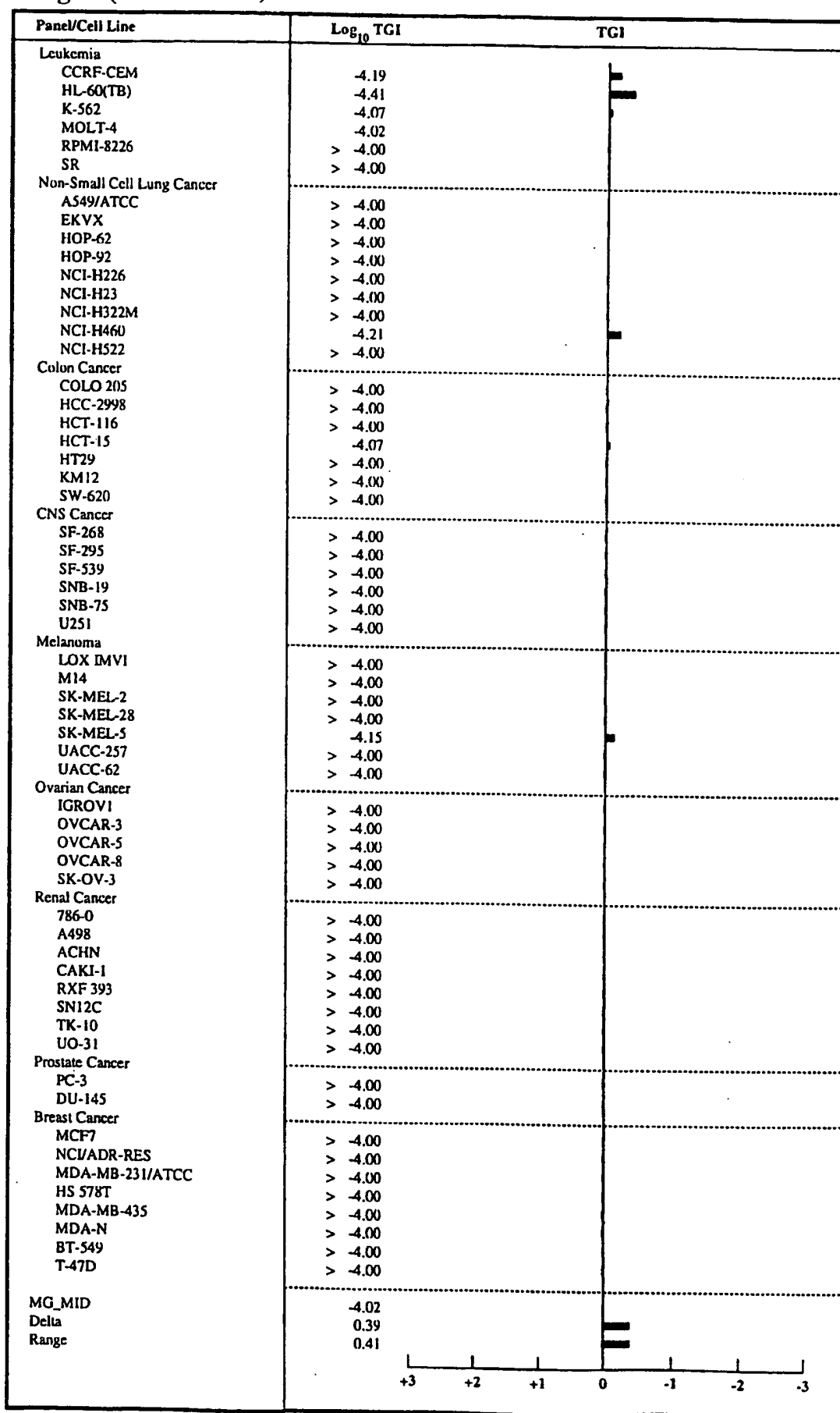


Fig. 8 (Sheet 3 of 3)

32/47

Panel/Cell Line	Log ₁₀ LC50	LC50
Leukemia		
CCRF-CEM	> -4.00	
HL-60(TB)	> -4.00	
K-562	> -4.00	
MOLT-4	> -4.00	
RPMI-8226	> -4.00	
SR	> -4.00	
Non-Small Cell Lung Cancer		
A549/ATCC	> -4.00	
EKVX	> -4.00	
HOP-62	> -4.00	
HOP-92	> -4.00	
NCI-H226	> -4.00	
NCI-H23	> -4.00	
NCI-H322M	> -4.00	
NCI-H460	> -4.00	
NCI-H522	> -4.00	
Colon Cancer		
COLO 205	> -4.00	
HCC-2998	> -4.00	
HCT-116	> -4.00	
HCT-15	> -4.00	
HT29	> -4.00	
KM12	> -4.00	
SW-620	> -4.00	
CNS Cancer		
SF-268	> -4.00	
SF-295	> -4.00	
SF-539	> -4.00	
SNB-19	> -4.00	
SNB-75	> -4.00	
U251	> -4.00	
Melanoma		
LOX IMVI	> -4.00	
M14	> -4.00	
SK-MEL-2	> -4.00	
SK-MEL-28	> -4.00	
SK-MEL-5	> -4.00	
UACC-257	> -4.00	
UACC-62	> -4.00	
Ovarian Cancer		
IGROV1	> -4.00	
OVCAR-3	> -4.00	
OVCAR-5	> -4.00	
OVCAR-8	> -4.00	
SK-OV-3	> -4.00	
Renal Cancer		
786-0	> -4.00	
A498	> -4.00	
ACHN	> -4.00	
CAKI-1	> -4.00	
RXF 393	> -4.00	
SN12C	> -4.00	
TK-10	> -4.00	
UO-31	> -4.00	
Prostate Cancer		
PC-3	> -4.00	
DU-145	> -4.00	
Breast Cancer		
MCF7	> -4.00	
NCI/ADR-RES	> -4.00	
MDA-MB-231/ATCC	> -4.00	
HS 578T	> -4.00	
MDA-MB-435	> -4.00	
MDA-N	> -4.00	
BT-549	> -4.00	
T-47D	> -4.00	
MG_MID	-4.00	
Delta	0.00	
Range	0.00	

+3 +2 +1 0 -1 -2 -3

33/47

Fig. 9 (A)

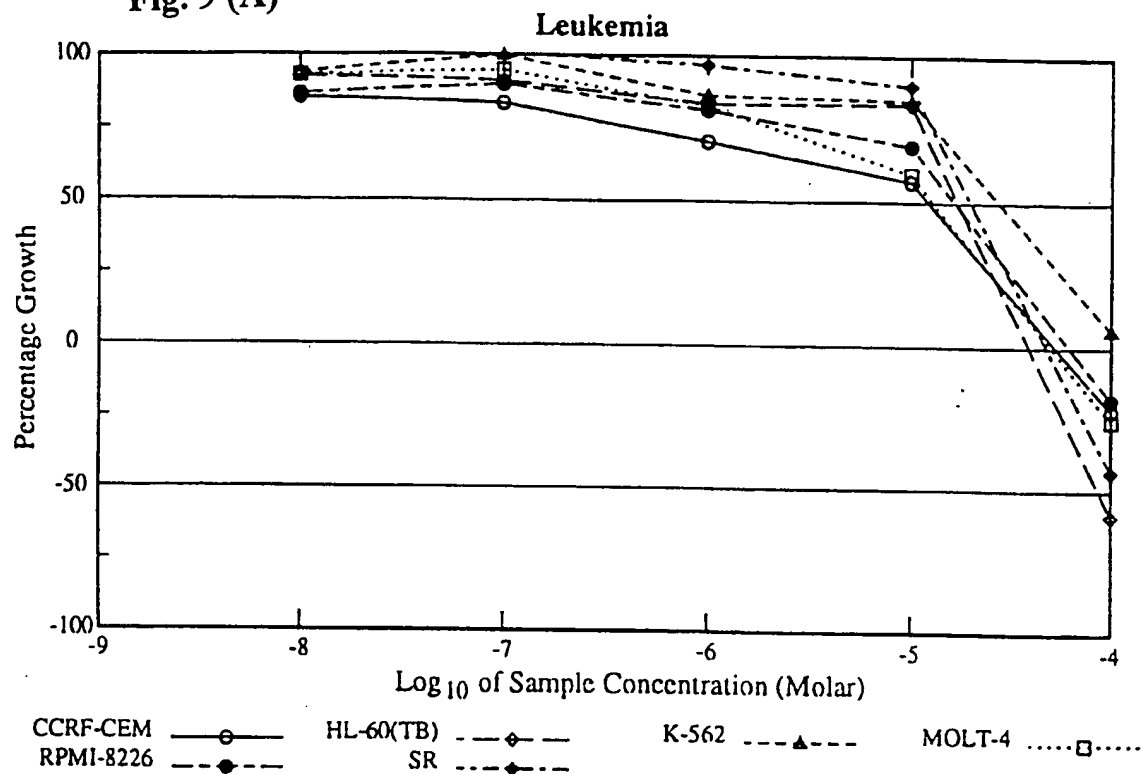
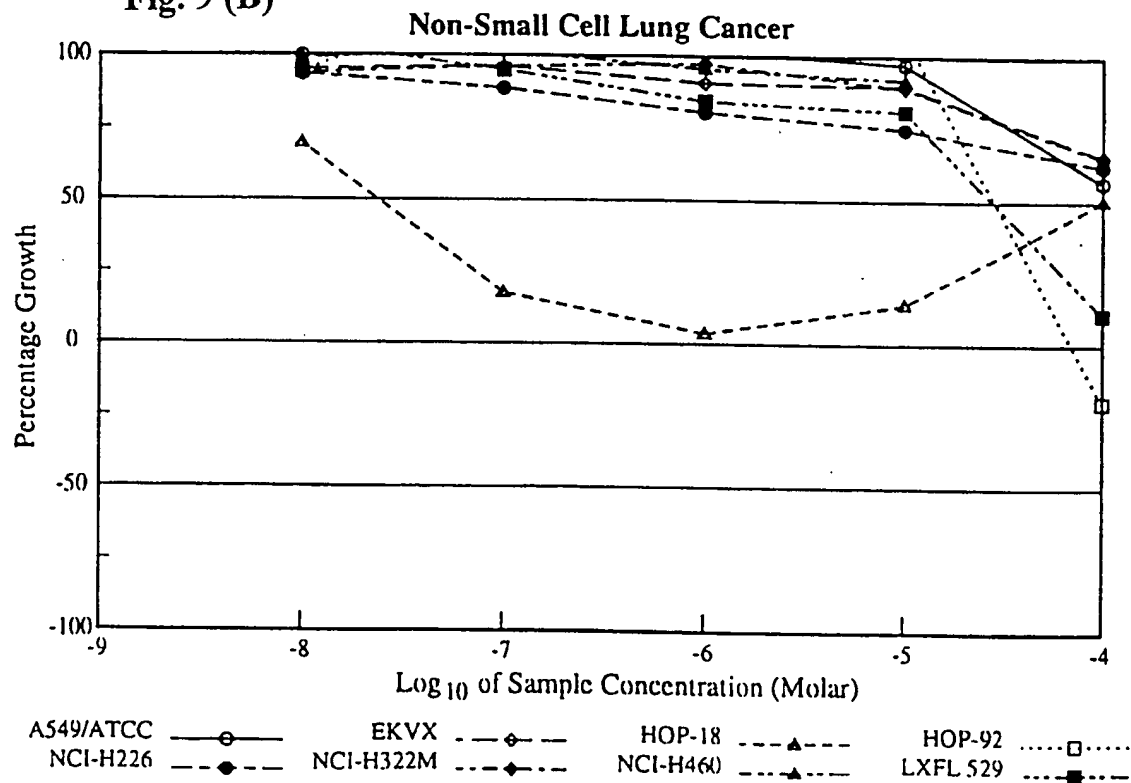


Fig. 9 (B)



34/47

Fig. 9 (C)

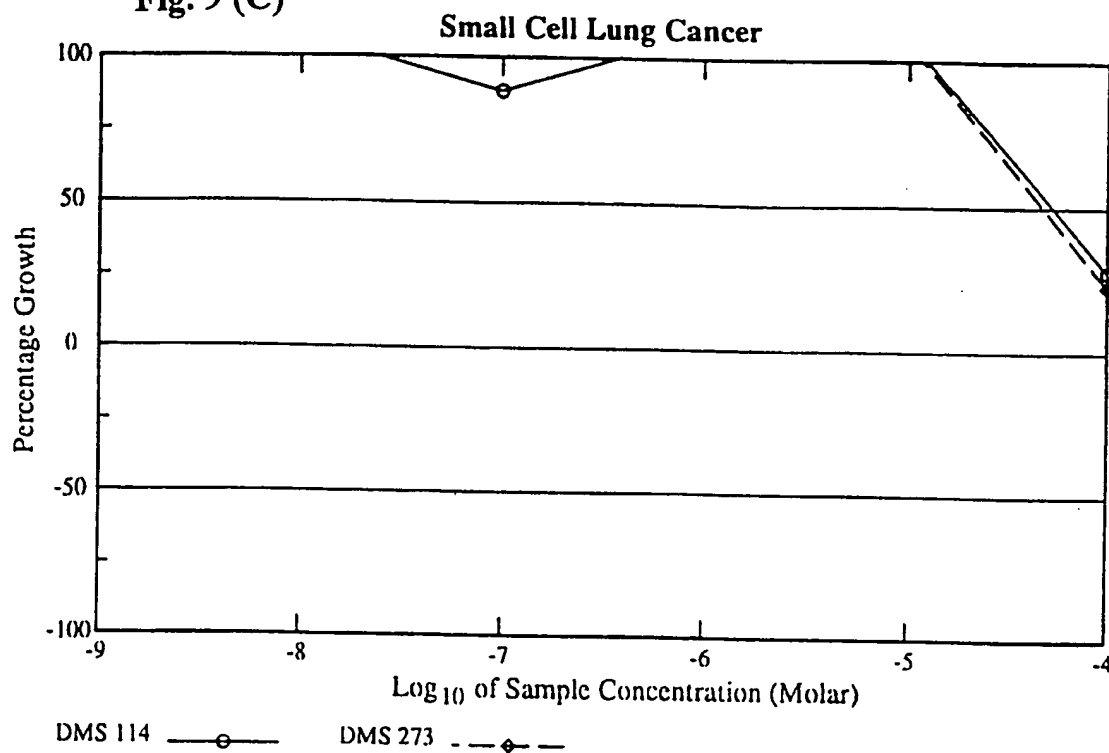
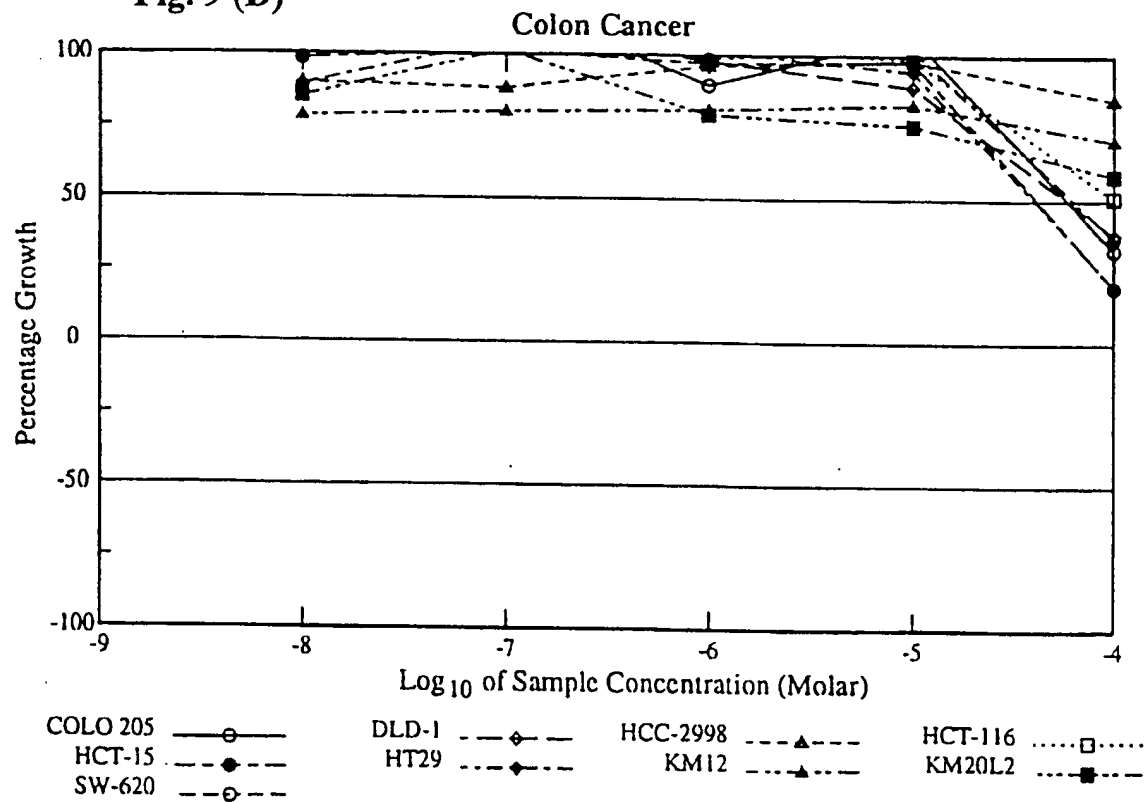


Fig. 9 (D)



35/47

Fig. 9 (E)

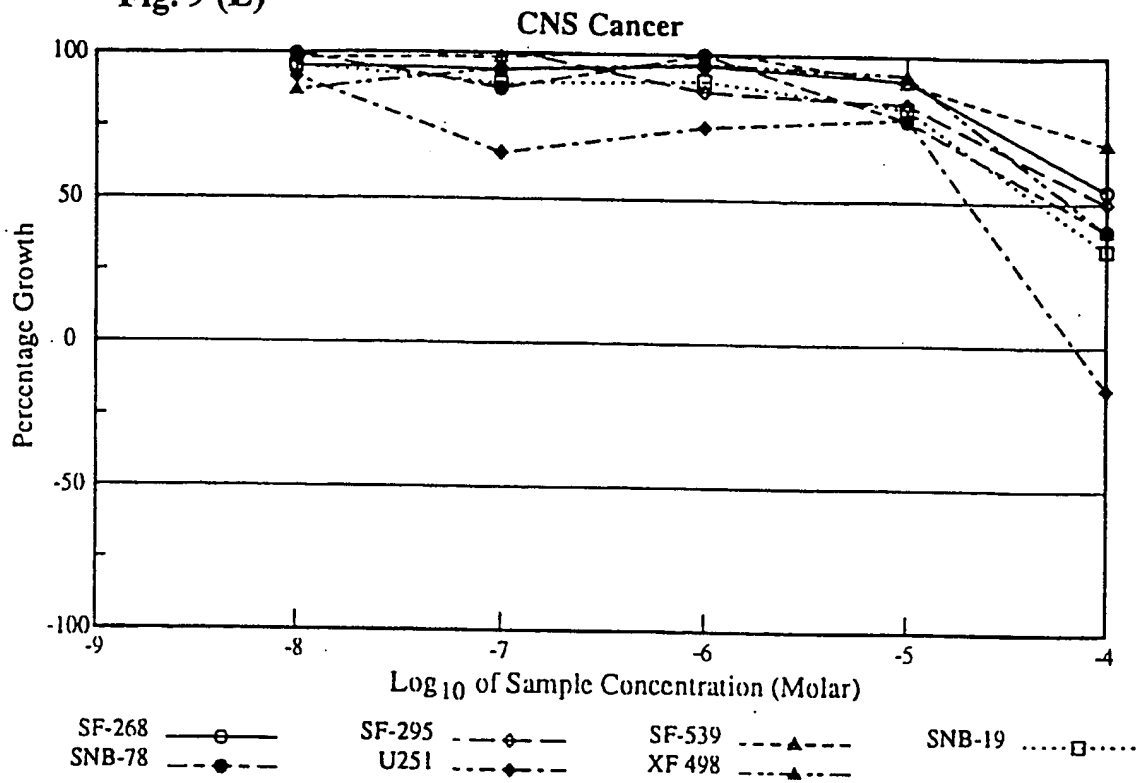
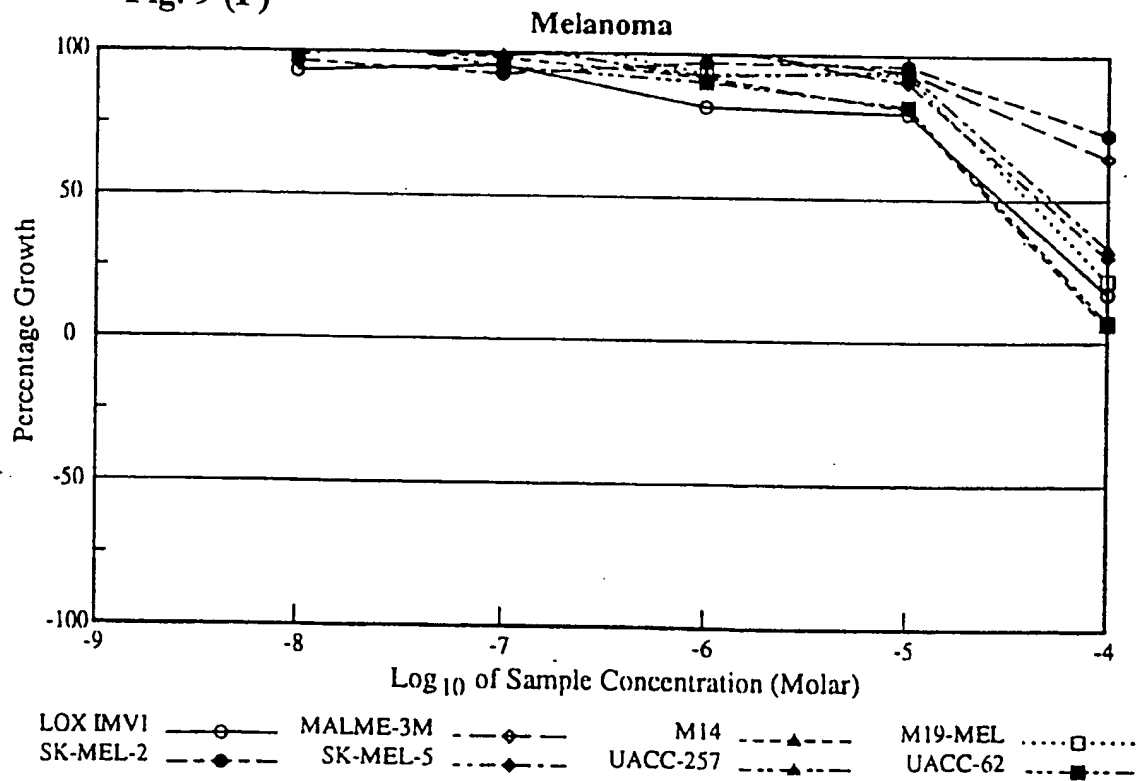


Fig. 9 (F)



36/47

Fig. 9 (G)

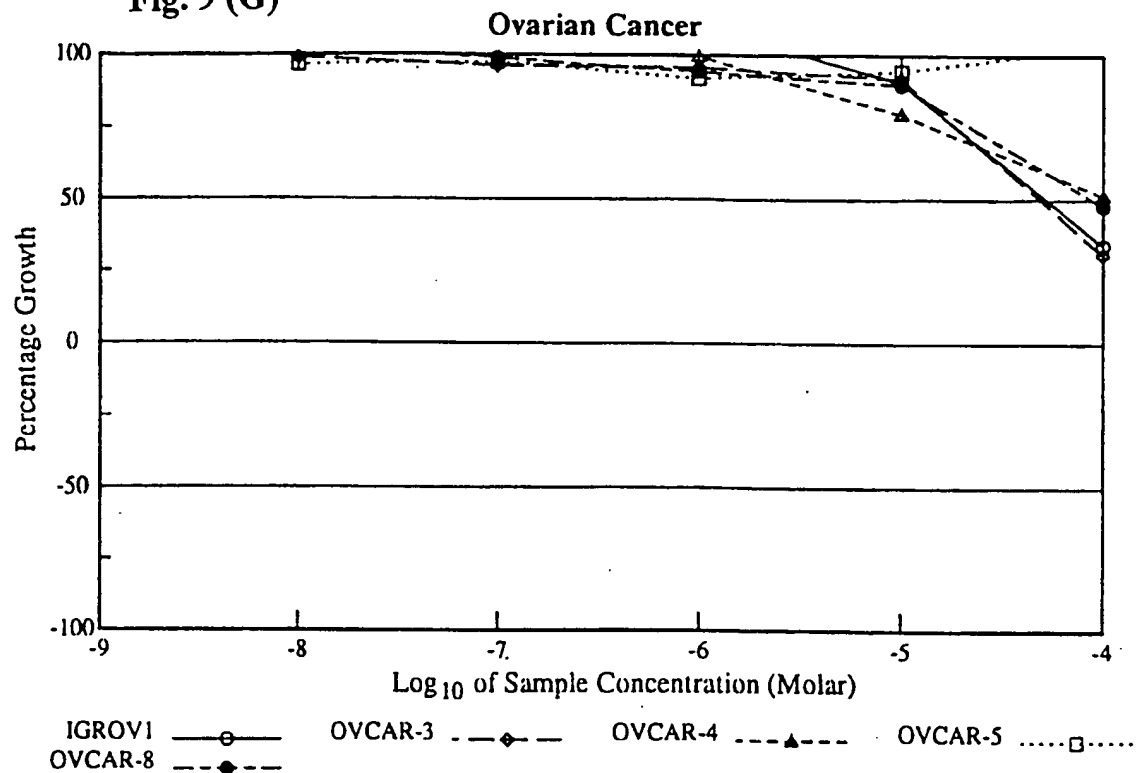


Fig. 9 (H)

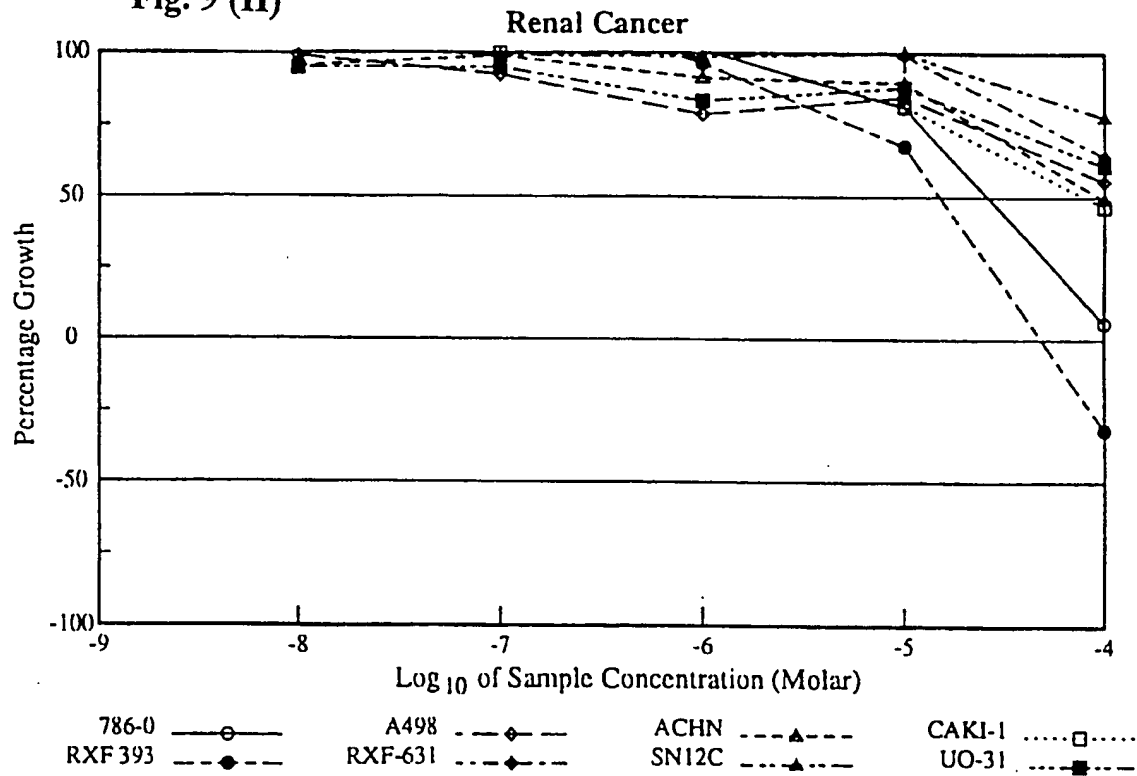


Fig. 10 (Sheet 1 of 3)

37/47

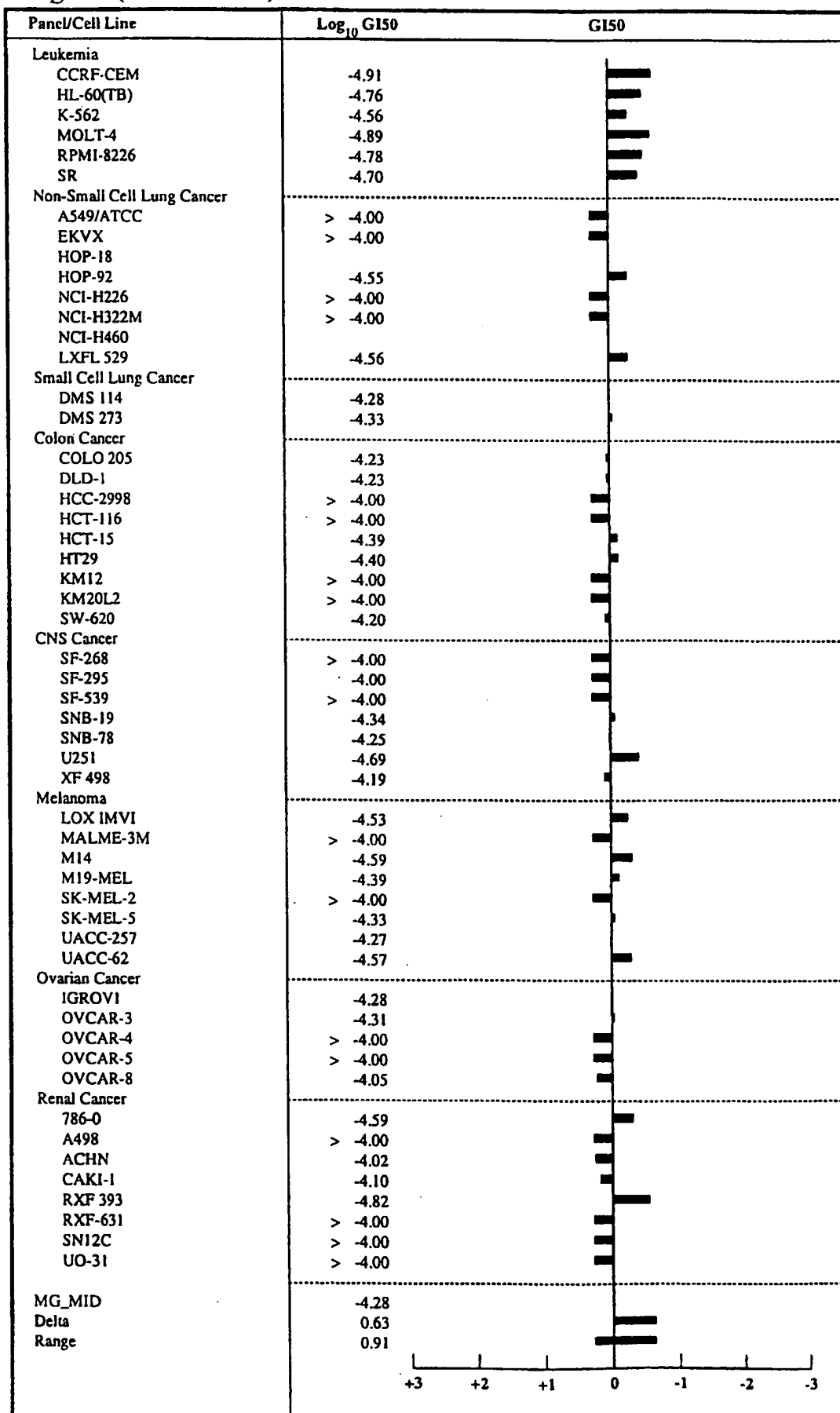


Fig. 10 (Sheet 2 of 3)

38/47

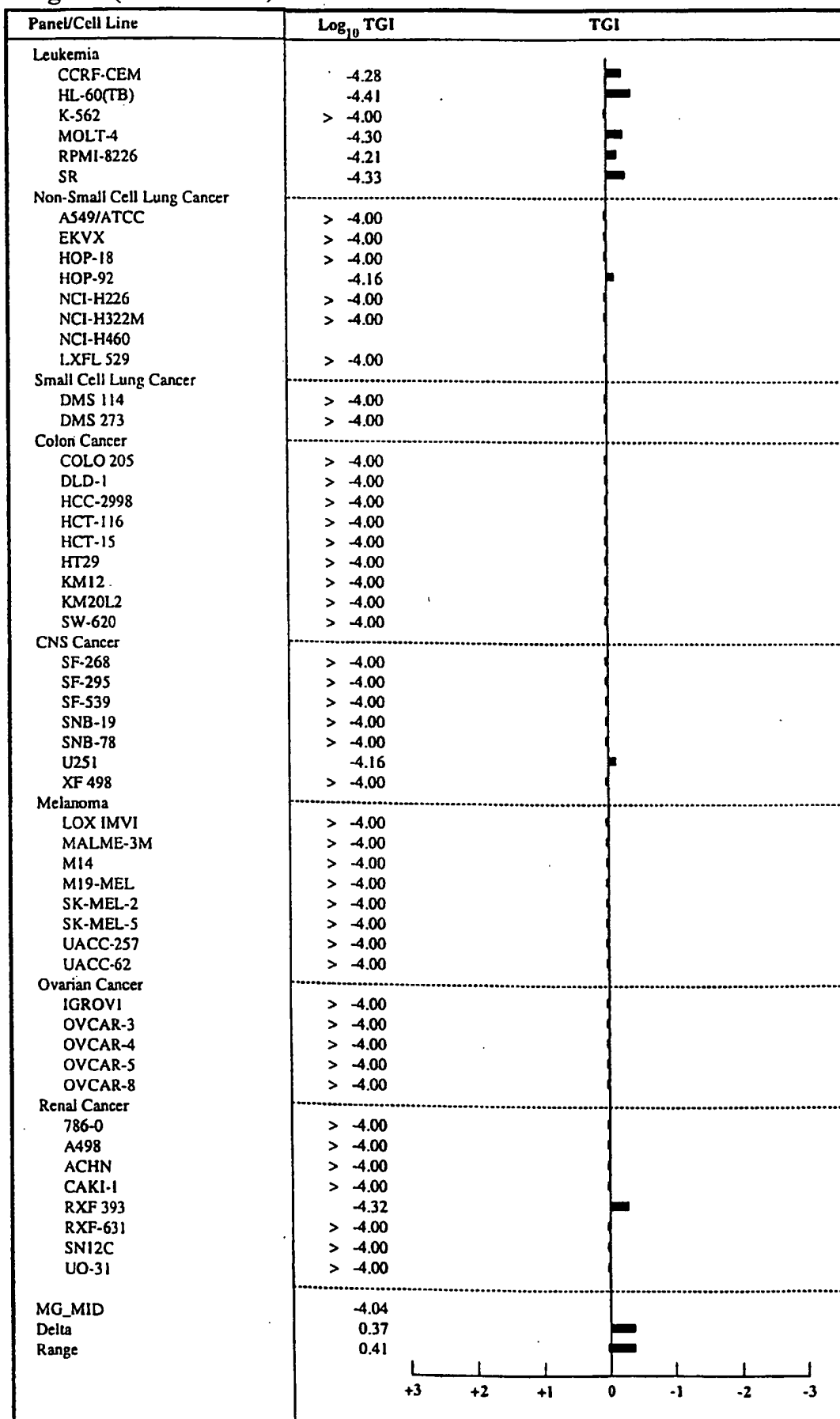


Fig. 10 (Sheet 3 of 3)

39/47

Panel/Cell Line	Log ₁₀ LC50	LC50
Leukemia		
CCRF-CEM	> -4.00	
HL-60(TB)	-4.06	
K-562	> -4.00	
MOLT-4	> -4.00	
RPMI-8226	> -4.00	
SR	> -4.00	
Non-Small Cell Lung Cancer		
A549/ATCC	> -4.00	
EKVX	> -4.00	
HOP-18	> -4.00	
HOP-92	> -4.00	
NCI-H226	> -4.00	
NCI-H322M	> -4.00	
NCI-H460		
LXFL 529	> -4.00	
Small Cell Lung Cancer		
DMS 114	> -4.00	
DMS 273	> -4.00	
Colon Cancer		
COLO 205	> -4.00	
DLD-1	> -4.00	
HCC-2998	> -4.00	
HCT-116	> -4.00	
HCT-15	> -4.00	
HT29	> -4.00	
KM12	> -4.00	
KM20L2	> -4.00	
SW-620	> -4.00	
CNS Cancer		
SF-268	> -4.00	
SF-295	> -4.00	
SF-539	> -4.00	
SNB-19	> -4.00	
SNB-78	> -4.00	
U251	> -4.00	
XF 498	> -4.00	
Melanoma		
LOX IMVI	> -4.00	
MALME-3M	> -4.00	
M14	> -4.00	
M19-MEL	> -4.00	
SK-MEL-2	> -4.00	
SK-MEL-5	> -4.00	
UACC-257	> -4.00	
UACC-62	> -4.00	
Ovarian Cancer		
IGROV1	> -4.00	
OVCAR-3	> -4.00	
OVCAR-4	> -4.00	
OVCAR-5	> -4.00	
OVCAR-8	> -4.00	
Renal Cancer		
786-O	> -4.00	
A498	> -4.00	
ACHN	> -4.00	
CAKI-1	> -4.00	
RXF 393	> -4.00	
RXF-631	> -4.00	
SN12C	> -4.00	
UO-31	> -4.00	
MG_MID	-4.00	
Delta	0.06	
Range	0.06	

+3 +2 +1 0 -1 -2 -3

40/47

Fig. 11 (A)

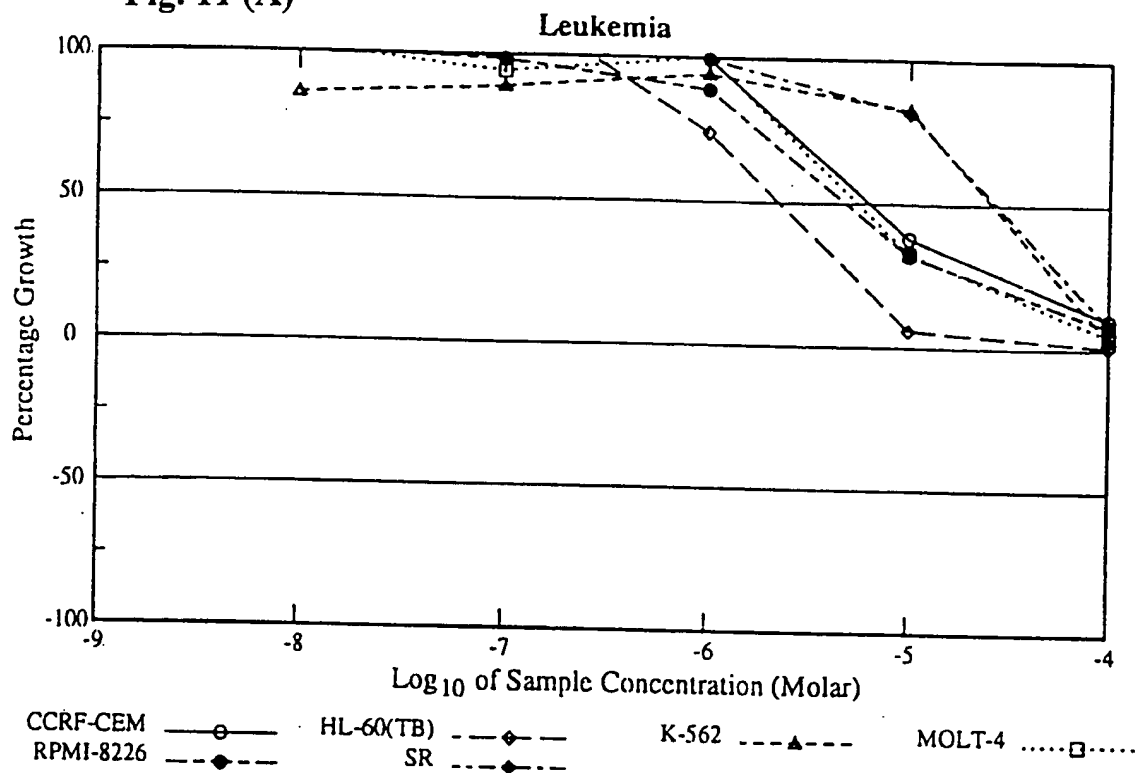
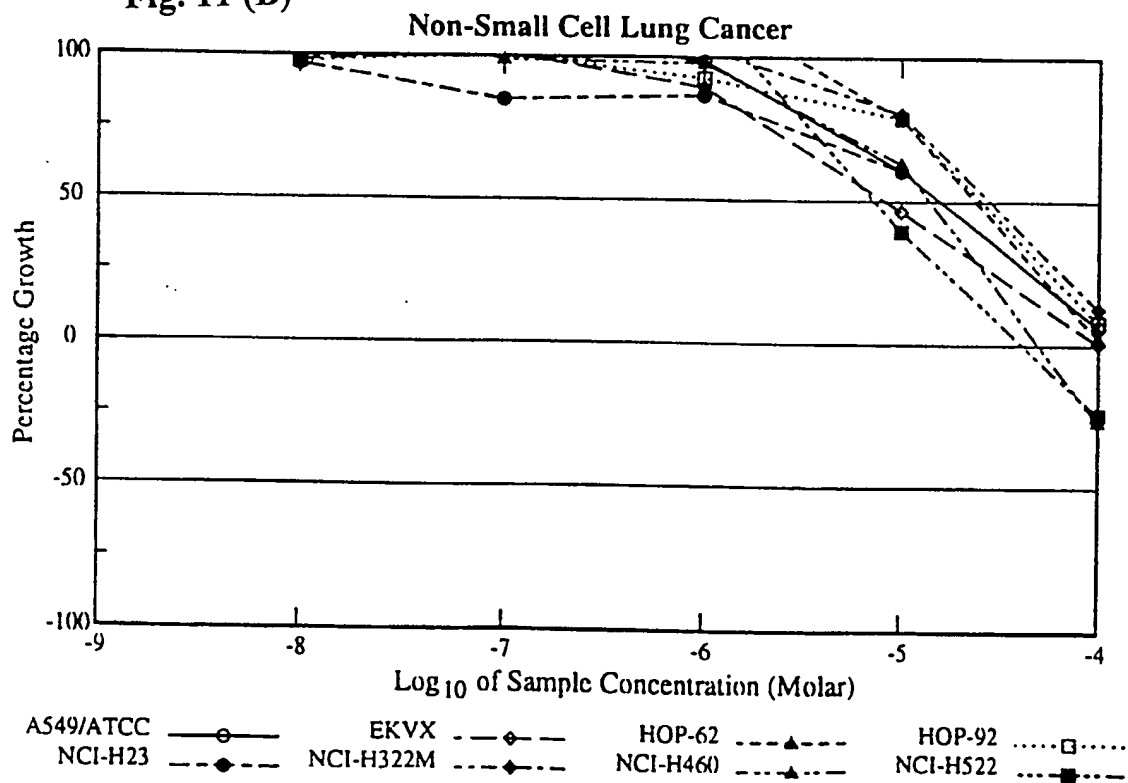


Fig. 11 (B)



41/47

Fig. 11 (C)

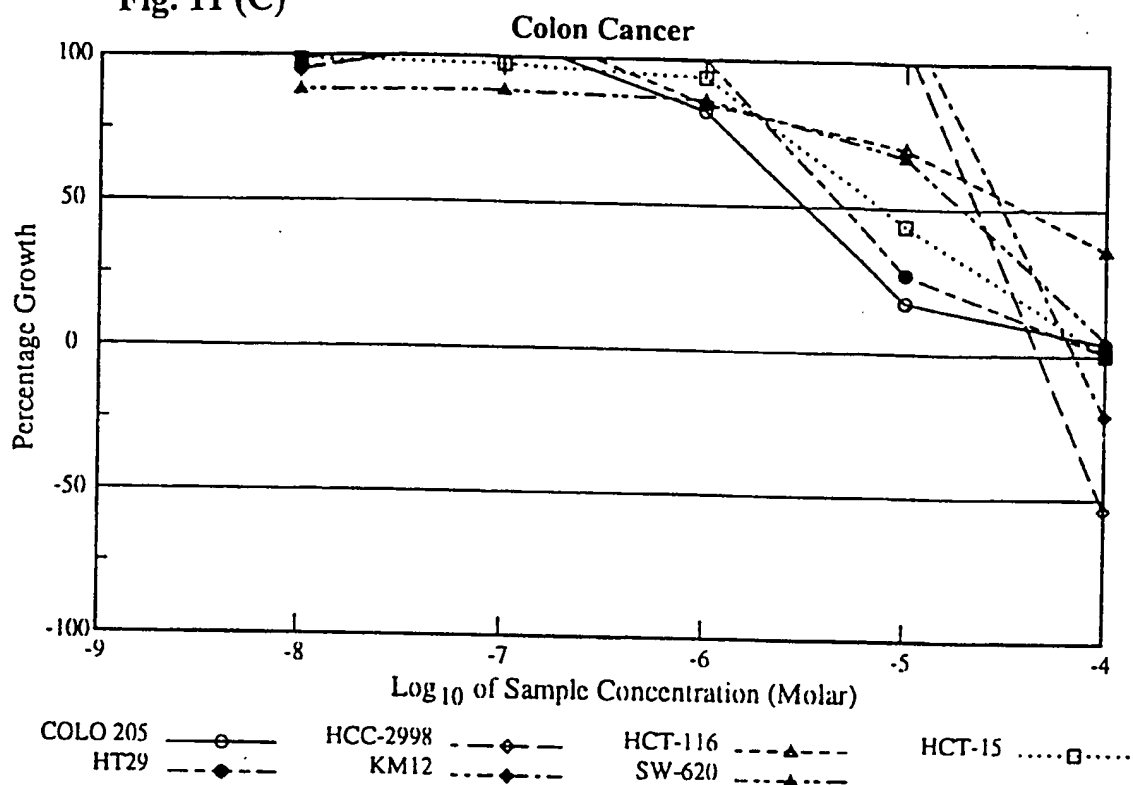
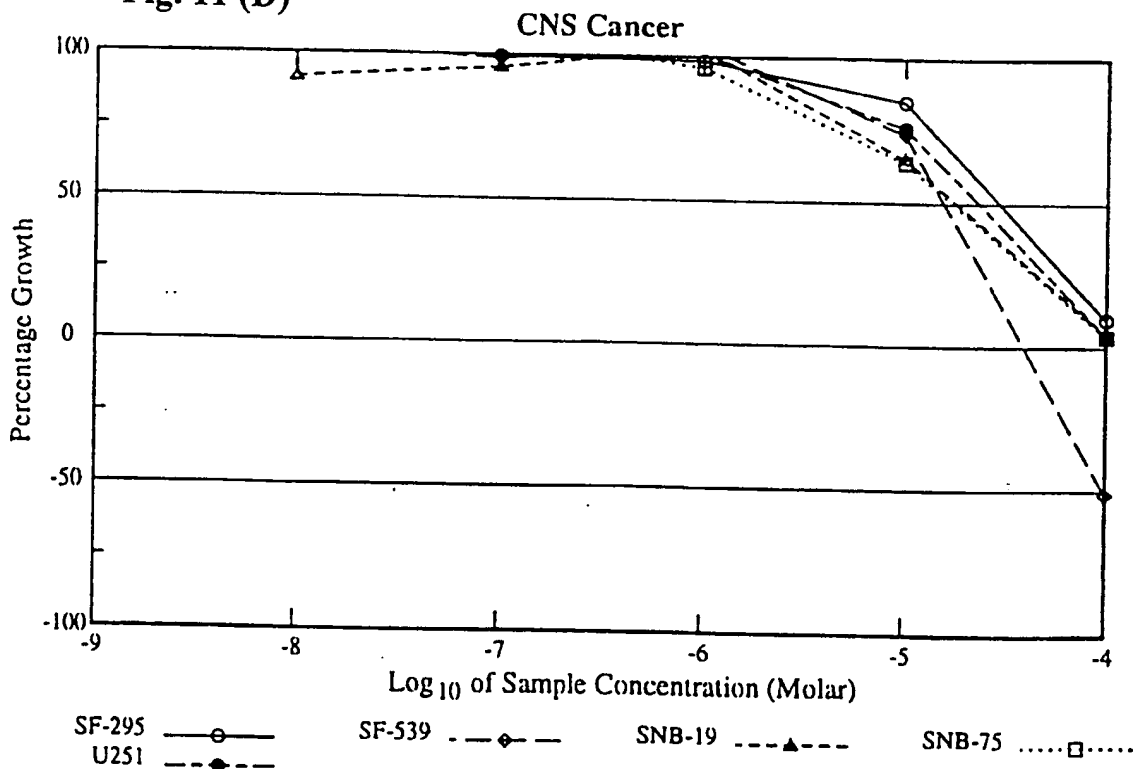


Fig. 11 (D)



42/47

Fig. 11 (E)

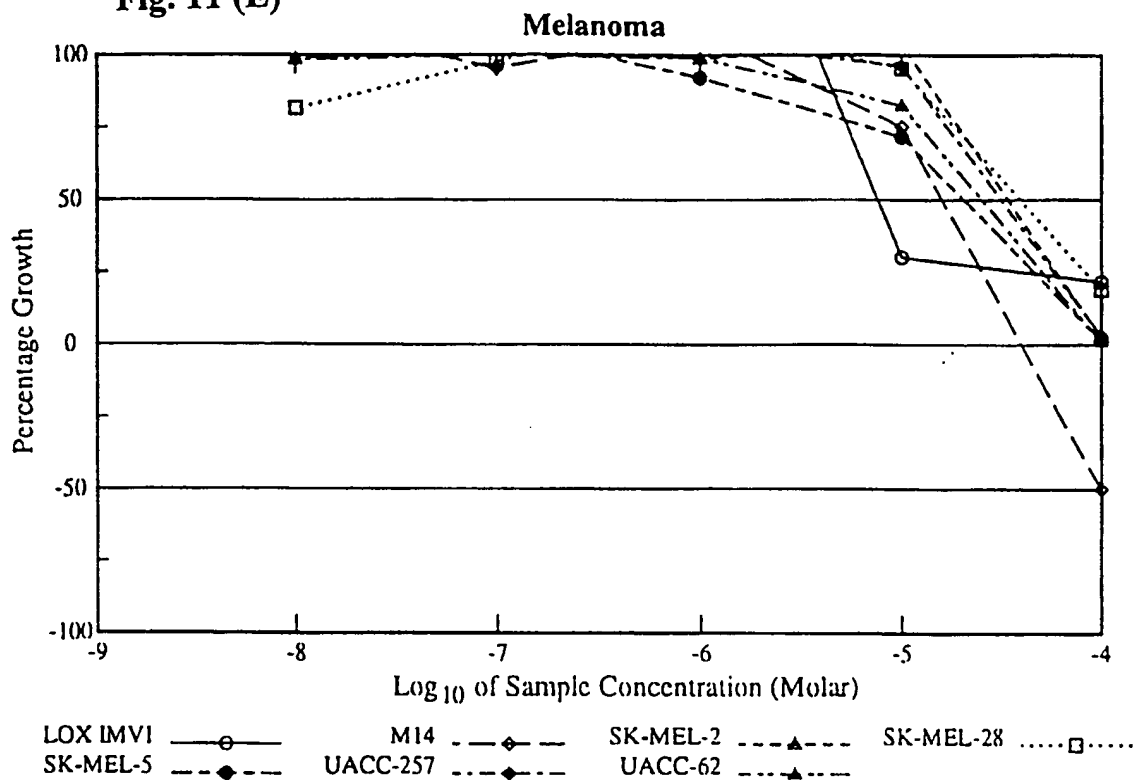
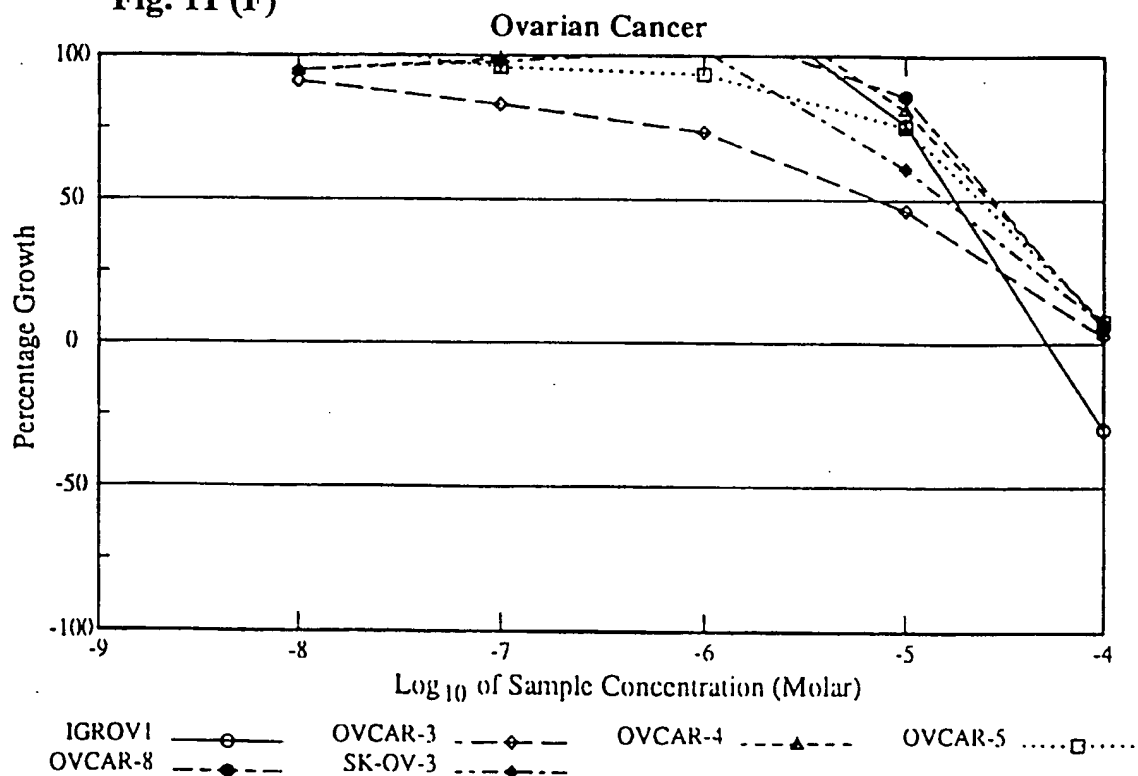


Fig. 11 (F)



43/47

Fig. 11 (G)

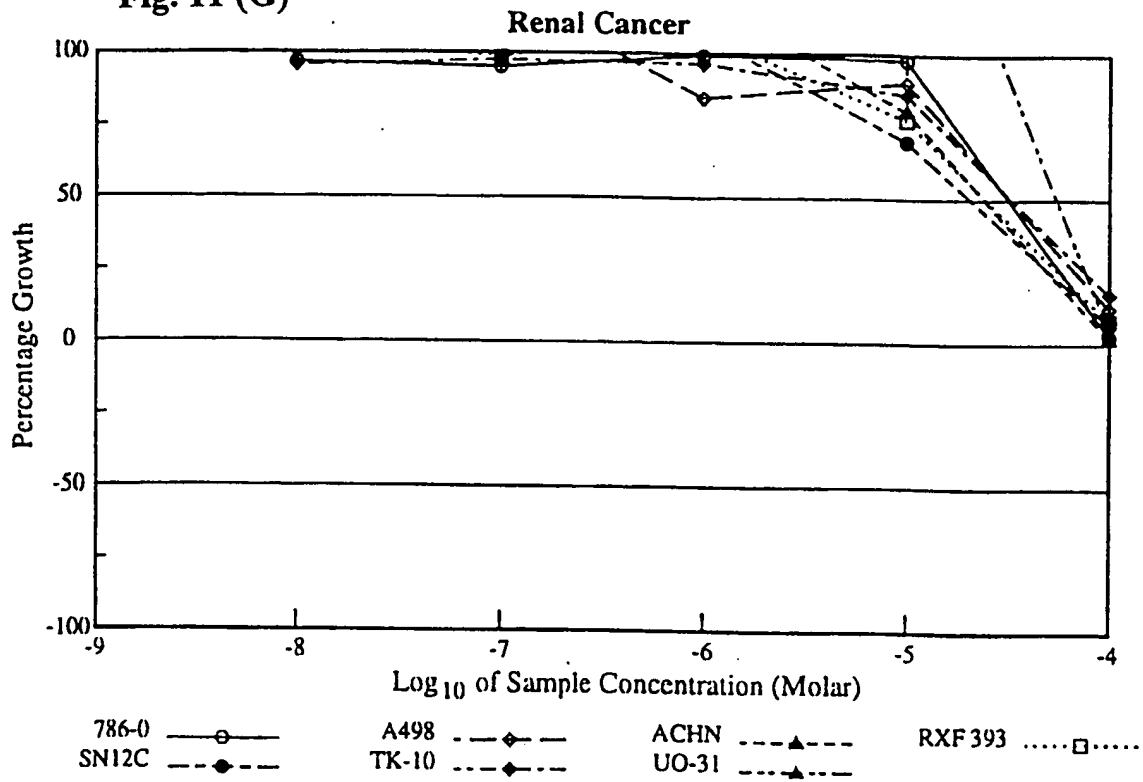
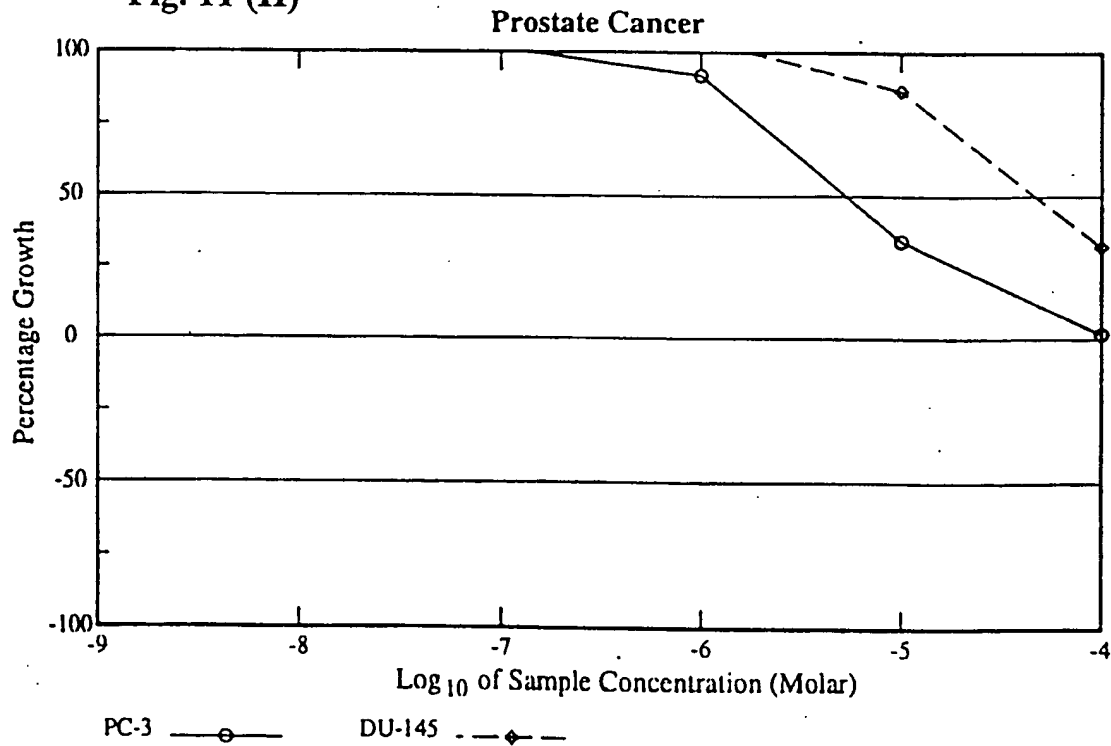


Fig. 11 (H)



44/47

Fig. 11 (I)

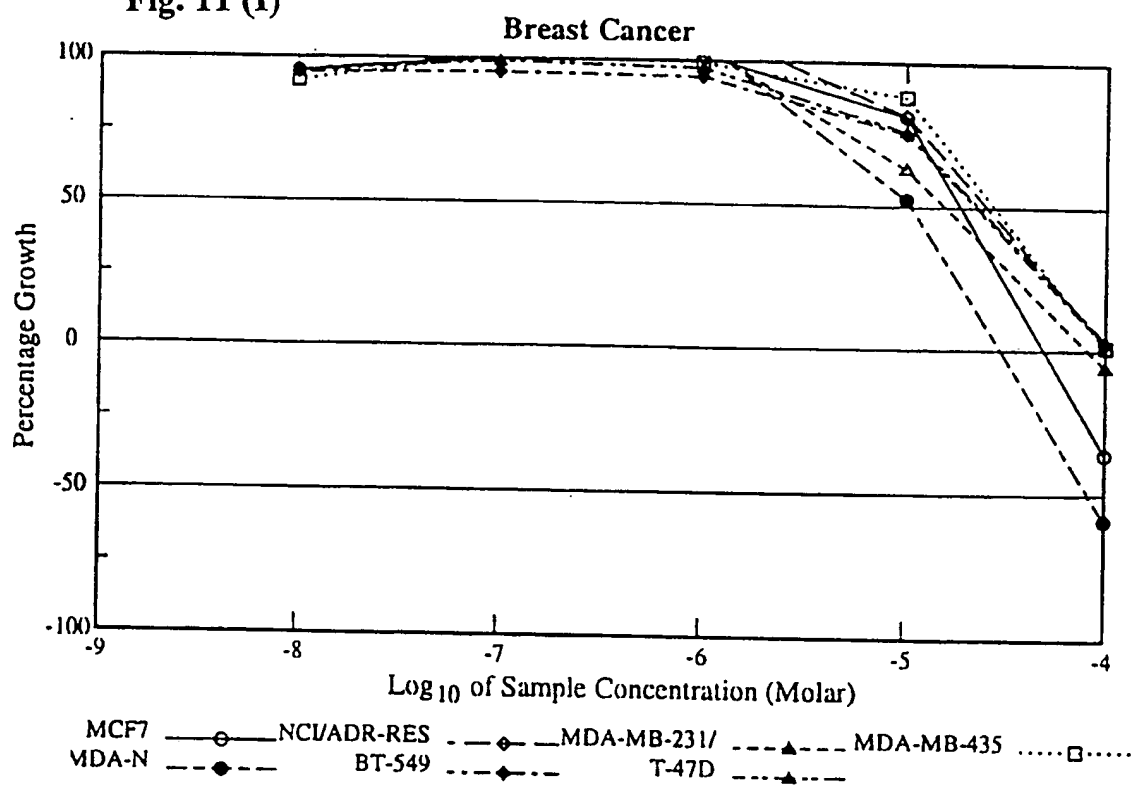


Fig. 12 (Sheet 1 of 3)

45/47

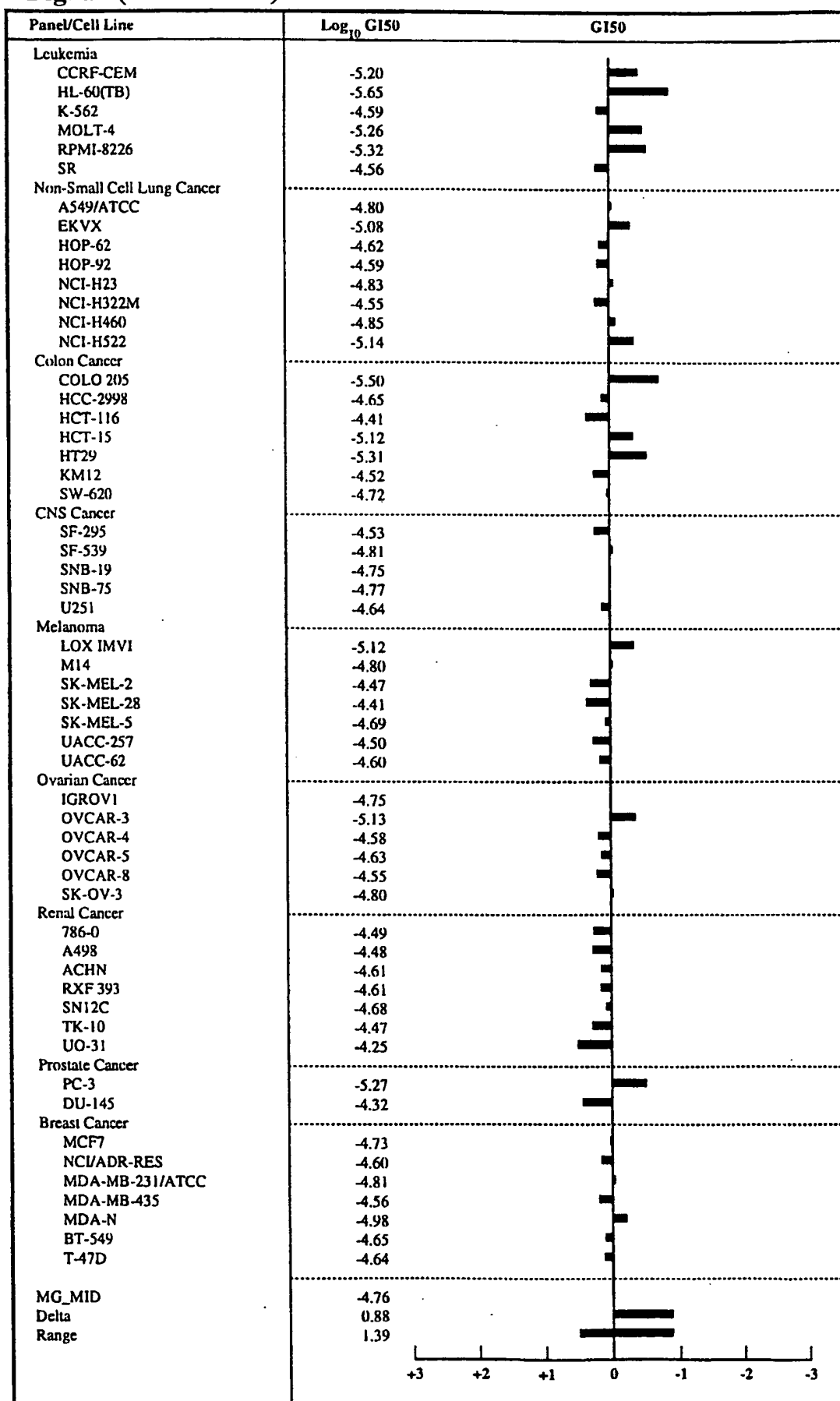


Fig. 12 (Sheet 2 of 3) 46/47

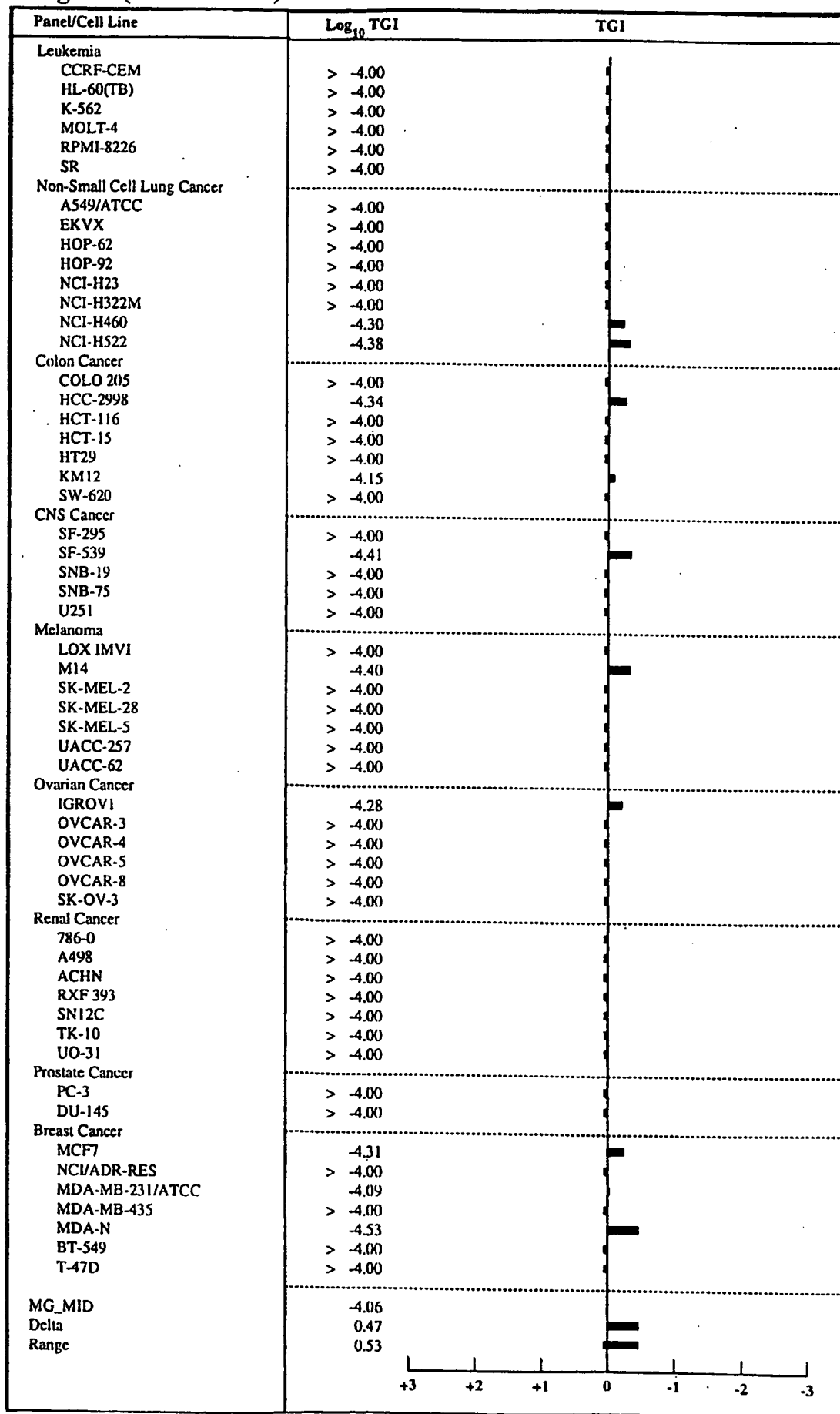


Fig. 12 (Sheet 3 of 3)

47/47

Panel/Cell Line	Log ₁₀ LC50	LC50
Leukemia		
CCRF-CEM	> -4.00	
HL-60(TB)	> -4.00	
K-562	> -4.00	
MOLT-4	> -4.00	
RPMI-8226	> -4.00	
SR	> -4.00	
Non-Small Cell Lung Cancer		
A549/ATCC	> -4.00	
EKVX	> -4.00	
HOP-62	> -4.00	
HOP-92	> -4.00	
NCI-H23	> -4.00	
NCI-H322M	> -4.00	
NCI-H460	> -4.00	
NCI-H522	> -4.00	
Colon Cancer		
COLO 205	> -4.00	
HCC-2998	-4.02	
HCT-116	> -4.00	
HCT-15	> -4.00	
HT29	> -4.00	
KM12	> -4.00	
SW-620	> -4.00	
CNS Cancer		
SF-295	> -4.00	
SF-539	-4.01	
SNB-19	> -4.00	
SNB-75	> -4.00	
U251	> -4.00	
Melanoma		
LOX IMVI	> -4.00	
M14	> -4.00	
SK-MEL-2	> -4.00	
SK-MEL-28	> -4.00	
SK-MEL-5	> -4.00	
UACC-257	> -4.00	
UACC-62	> -4.00	
Ovarian Cancer		
IGROV1	> -4.00	
OVCAR-3	> -4.00	
OVCAR-4	> -4.00	
OVCAR-5	> -4.00	
OVCAR-8	> -4.00	
SK-OV-3	> -4.00	
Renal Cancer		
786-0	> -4.00	
A498	> -4.00	
ACHN	> -4.00	
RXF 393	> -4.00	
SN12C	> -4.00	
TK-10	> -4.00	
UO-31	> -4.00	
Prostate Cancer		
PC-3	> -4.00	
DU-145	> -4.00	
Breast Cancer		
MCF7	> -4.00	
NCI/ADR-RES	> -4.00	
MDA-MB-231/ATCC	> -4.00	
MDA-MB-435	> -4.00	
MDA-N	-4.08	
BT-549	> -4.00	
T-47D	> -4.00	
MG_MID	-4.00	
Delta	0.08	
Range	0.08	

+3 +2 +1 0 -1 -2 -3



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07D 219/08, A61K 31/435, 31/54, C07D 219/06, 219/04, 285/24, 285/16		A3	(11) International Publication Number: WO 98/49146
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(21) International Application Number: PCT/US98/08602 (22) International Filing Date: 28 April 1998 (28.04.98) (30) Priority Data: 60/044,256 28 April 1997 (28.04.97) US (71) Applicant (for all designated States except US): THE UNITED STATES OF AMERICA, represented by THE SECRETARY DEPARTMENT OF HEALTH & HUMAN SERVICES, THE NATIONAL INSTITUTES OF HEALTH [US/US]; Office of Technology Transfer, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852-3804 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): KELLEY, Michael, J. [US/US]; 8200 Gallery Court, Montgomery Village, MD 20886 (US). NAKAGAWA, Kazuhiko [JP/JP]; 1-12-9, Oonodai, Osakasayama, Osaka 589 (JP). DENT, Barry, Roy [NZ/NZ]; 1 Donald Crescent, Wellington 6005 (NZ). (74) Agent: SLATER, Stacey, C.; Klarquist, Sparkman, Campbell, Leigh & Whinston, LLP, One World Trade Center, Suite 1600, 121 S.W. Salmon Street, Portland, OR 97204-2988 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> (88) Date of publication of the international search report: 12 August 1999 (12.08.99)	
(54) Title: CYCLIN DEPENDENT KINASE (CDK)4 INHIBITORS AND THEIR USE FOR TREATING CANCER			
(57) Abstract <p>Certain derivatives of acridones and benzothiadiazines have been found to have anti-cancer properties by virtue of their specific inhibition of the cyclin D dependant kinase CDK4. These molecules inhibit CDK4 activity more than they inhibit the activity of other such kinases (e.g. CDC2 and CDK2). This specificity results in an improved therapeutic index when used as drugs to treat susceptible cancers.</p>			

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/08602

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07D219/08 A61K31/435 A61K31/54 C07D219/06 C07D219/04
C07D285/24 C07D285/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07D A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BALL K L ET AL: "CELL-CYCLE ARREST AND INHIBITION OF CDK4 ACTIVITY BY SMALL PEPTIDES BASED ON THE CARBOXY-TERMINAL DOMAIN OF P21WAF1" CURRENT BIOLOGY, vol. 7, no. 1, 1 January 1997, pages 71-80, XP002039815	1-57
X	FR 2 676 737 A (GROUPE ENSEIGNEMENT RECH CHIM) 27 November 1992 see the whole document --- -/--	1-21, 40-43



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone.
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

26 February 1999

Date of mailing of the international search report

28.05.99

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Authorized officer

Steendijk, M

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/08602

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>MANNANI R ET AL: "Preparation of 3,3'-linked bis(acridinones), 9,9'-linked bis(thioacridines) and 3-3', 9-9' bi-linked bis(thioacridinones)" EUR. J. MED. CHEM. (EJMCA5,02235234);91; VOL.26 (1); PP.117-19, XP002075295 UFR PHARM.;GROUPE ENSEIGN. RECH. CHIM. THER. ORG. PHYS.; MARSEILLE; 13385; FR. (FR) see the whole document</p>	1
X	<p>MANNANI R ET AL: "Synthesis of two novel thioacridine derivatives and comparison of their in vitro biological activities" CHEM.-BIOL. INTERACT. (CBINA8,00092797);90; VOL.74 (3); PP.291-303, XP002075296 FAC. PHARM.;GROUPE ETUD. RECH. CHIM. THER. ORG. PHYS.; MARSEILLE; FR. (FR) see the whole document</p>	1
X	<p>CHEMICAL ABSTRACTS, vol. 112, no. 19, 7 May 1990 Columbus, Ohio, US; abstract no. 178637g, MARTYNOVSKII ET AL.: "Synthesis , physicochemical properties and pharmacological activity of carbonyl-substituted 2-methoxy-9-thioacridines" XP002091482 & Izv. Timiryazevsk. S-kh. Akad., 1989, (5), 174-181 see abstract</p>	1-20,25
A	<p>CHEMICAL ABSTRACTS, vol. 070, no. 11, 17 March 1969 Columbus, Ohio, US; abstract no. 047270. WYSOCKA-SKRZELA B ET AL: "Tumor inhibiting compounds. XLIV. Syntheses of N-substituted 1-, 2-, 3-, and 4-methoxyacridones and thioacridones. 2" XP002075299 see abstract & ROCZ. CHEM. (ROCHAC);68; VOL.42 (10); PP.1755-61, POLSKA AKAD. NAUK;GDANSK; POLAND</p>	1-57
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/08602

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TAIT A ET AL: "Antitumor activity of methyl-4H-1,2,4-benzothiadiazin-3-yl-carbamodithioate-S,S-dioxide" BOLL. CHIM. FARM. (BCFAAI,00066648);90; VOL.129 (9); PP.273-5, XP002075297 UNIV. MODENA;DIP. SCI. FARM.; ITALY (IT)	2-14,39, 40
A	see the whole document	1,15-38, 41-57
X	CHEMICAL ABSTRACTS, vol. 124, no. 17, 22 April 1996 Columbus, Ohio, US; abstract no. 232400u, PARK ET AL.: "Synthesis and cytotoxic properties of 3,4-dihydro-3-oxo-2H-1,2,4-benzothiadiazine 1,1-dioxides" XP002075300	2-14,39, 40
A	& Yakhak Hoechi, 1995, 39(6), 631-635 see abstract	1,15-38, 41-57
X	DE 33 31 459 A (FINK MICHAEL DR MED;BERGER MARTIN DR MED) 1 March 1984 see the whole document	2-14,39, 40
Y,P	WO 97 49692 A (NOVONORDISK AS) 31 December 1997 see the whole document	2-14,39, 40
X,Y	CHEMICAL ABSTRACTS, vol. 120, no. 25, 20 June 1994 Columbus, Ohio, US; abstract no. 315309, LEE ET AL.: "Inhibition of cell growth by K ⁺ channel modulators is due to interference with agonist-induced Ca ²⁺ release" XP002091483 & Cell. Signalling, 1993, 5(6), 803-809 see abstract	2-14,39, 40
X	CHEMICAL ABSTRACTS, vol. 73, no. 15, 12 October 1970 Columbus, Ohio, US; abstract no. 75517, NAKAHARA ET AL.: "Oncostatic activities of some fluoro compounds against Ehrlich carcinoma in mice" XP002091484 & Wakayama Daigaku Gakugeigakubu Kiyo, Shizen kagaku 1968, no. 18, 15-17 see abstract	2-14,39, 40
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/08602

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>REDDY ET AL.: "Synthesis & biological activity of 3-pyrazolyl-4-substituted-2H-1,2,4-benzothiadiazine 1,1-dioxides" INDIAN JOURNAL OF CHEMISTRY, vol. 24B, 1985, pages 1295-1297, XP002091480 see the whole document</p> <p style="text-align: center;">---</p>	2-14, 39, 40
X	<p>JIANG ET AL.: "Synthesis and biological evaluation of 2-styrylquinazolin-4(3H)-ones, a new class of antimitotic anticancer agents which inhibit tubulin polymerization" J.MED.CHEM., vol. 33, 1990, pages 1721-1728, XP002091481 see page 1723; table IV</p> <p style="text-align: center;">---</p>	2-14, 39, 40
X	<p>CHEMICAL ABSTRACTS, vol. 113, no. 13, 24 September 1990 Columbus, Ohio, US; abstract no. 115267, SADANA ETAL.: "Antibacterial activity and synthesis of 4-(substituted benzylamino)-1,2,3-benzothiadiazine 1,1-dioxides" XP002091485 & Indian J. Chem., Sect. B, 1990, 29b(6), 598-599. RN: 129226-06-8 see abstract</p> <p style="text-align: center;">---</p>	2-20, 36-38
X	<p>US 3 090 783 A (YALE) 21 May 1963 see the whole document</p> <p style="text-align: center;">-----</p>	2-20, 29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/08602

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☒ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1,21,28,43,50 and (part) 2-20, 22-27,29-42,44-49,
51-57

Compounds, antineoplastic compositions and methods involving at least one thioacridone, possibly in combination with a benzothiadiazine.

2. Claims: 2-20, 22-27,29-42,44-49,51-57 (all part)

Antineoplastic compositions and methods involving a benzothiadiazine, not in combination with thioacridinones

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/08602

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR 2676737 A	27-11-1992	NONE	
DE 3331459 A	01-03-1984	NONE	
WO 9749692 A	31-12-1997	AU 3166497 A	14-01-1998
US 3090783 A	21-05-1963	NONE	